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Twenty-Ninth Annual Meeting
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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
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4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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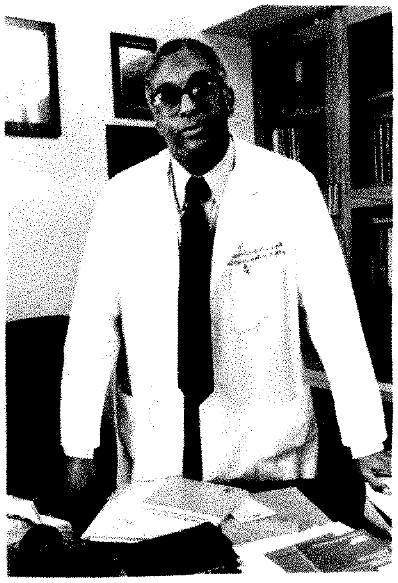

"If everyone over 50 had checkups for colorectal cancer, the cure rate could be as high as 75%," says Dr. LaSalle D. Leffall, Jr., past president, American Cancer Society. "You can't cure it if you don't know you have it." But if it's detected early, the cure rate for colorectal cancer is very high. Your doctor can perform the digital and proctoscopic exams, and you take care of the simple stool blood test at home.

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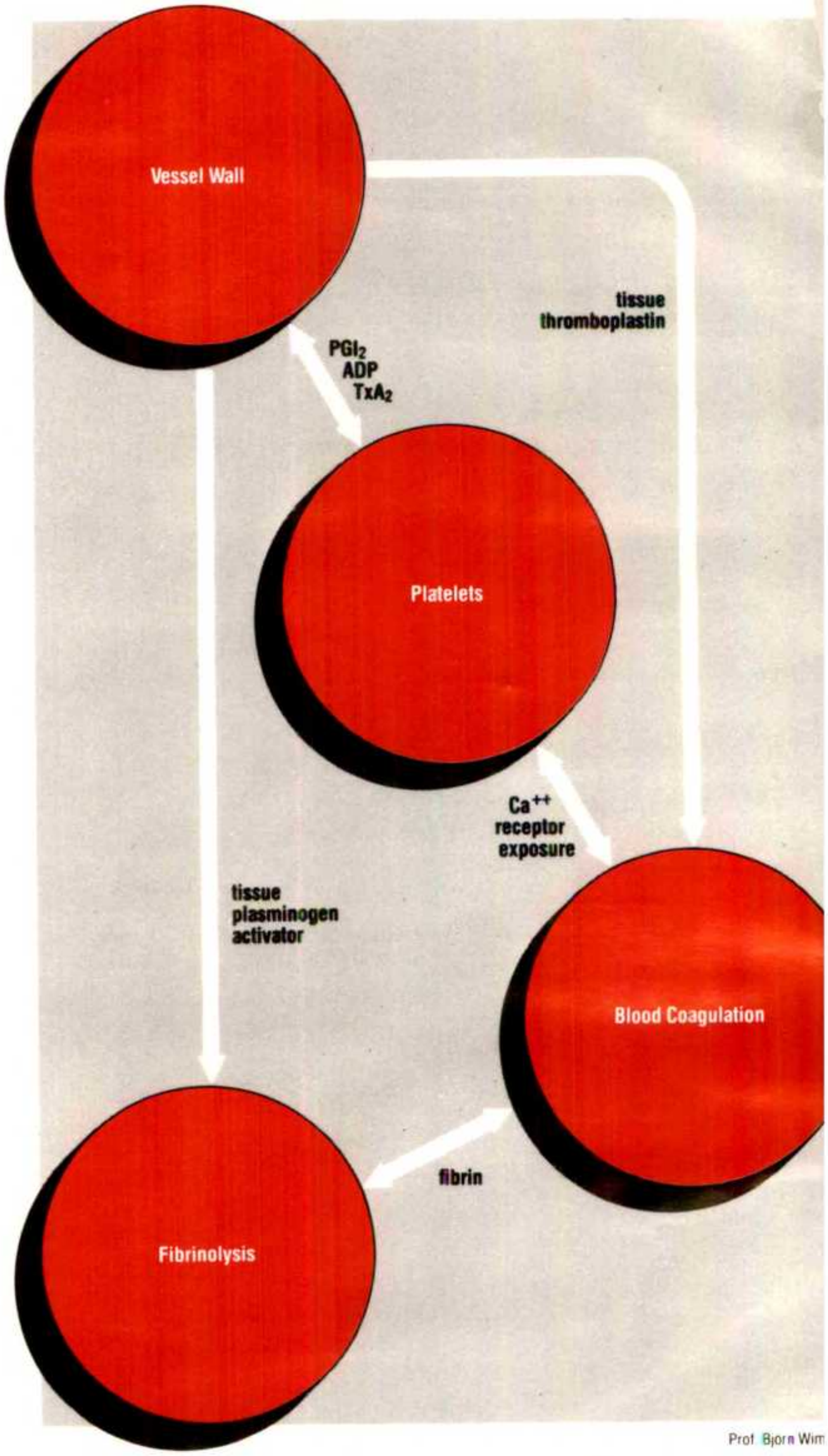

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All manuscripts submitted for consideration for publication in BLOOD should be sent to: John W. Adamson, MD, Editor, BLOOD, University District Building—Room 410, 1107 NE 45th St, Seattle, WA 98105. Telephone: (206) 545-8080. Please note that a processing fee of $50.00 must accompany each manuscript submitted for publication in BLOOD.

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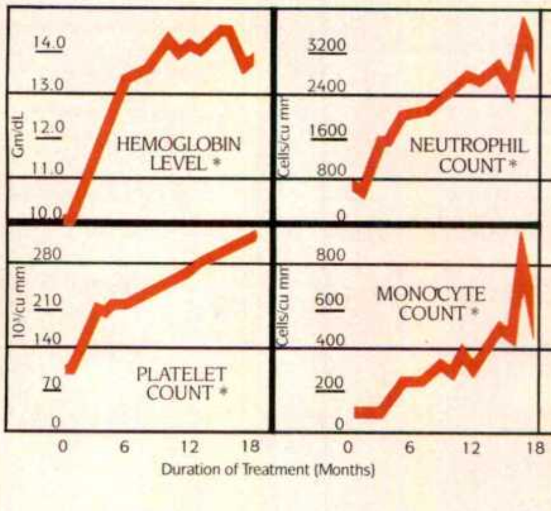

- Virtually all patients benefit: 89% improved, 11% stabilized.
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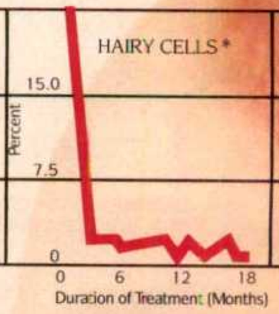

- As blood parameters normalize, there is a concurrent decrease in peripheral blood hairy cells.

The Roferon-A multicenter study consisted of 96 patients, all of whom were evaluated for safety. Seventy-five patients were evaluable for efficacy. Of these, 57 had prior splenectomy and 18 had not; many were pancytopenic and transfusion-dependent. Of the 21 patients not evaluable for efficacy, 16 were not on drug long enough for efficacy evaluation at the time of data analysis, 2 were removed for administrative reasons, and 3 were removed for preexisting intercurrent illness.

*Multicenter Study, Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

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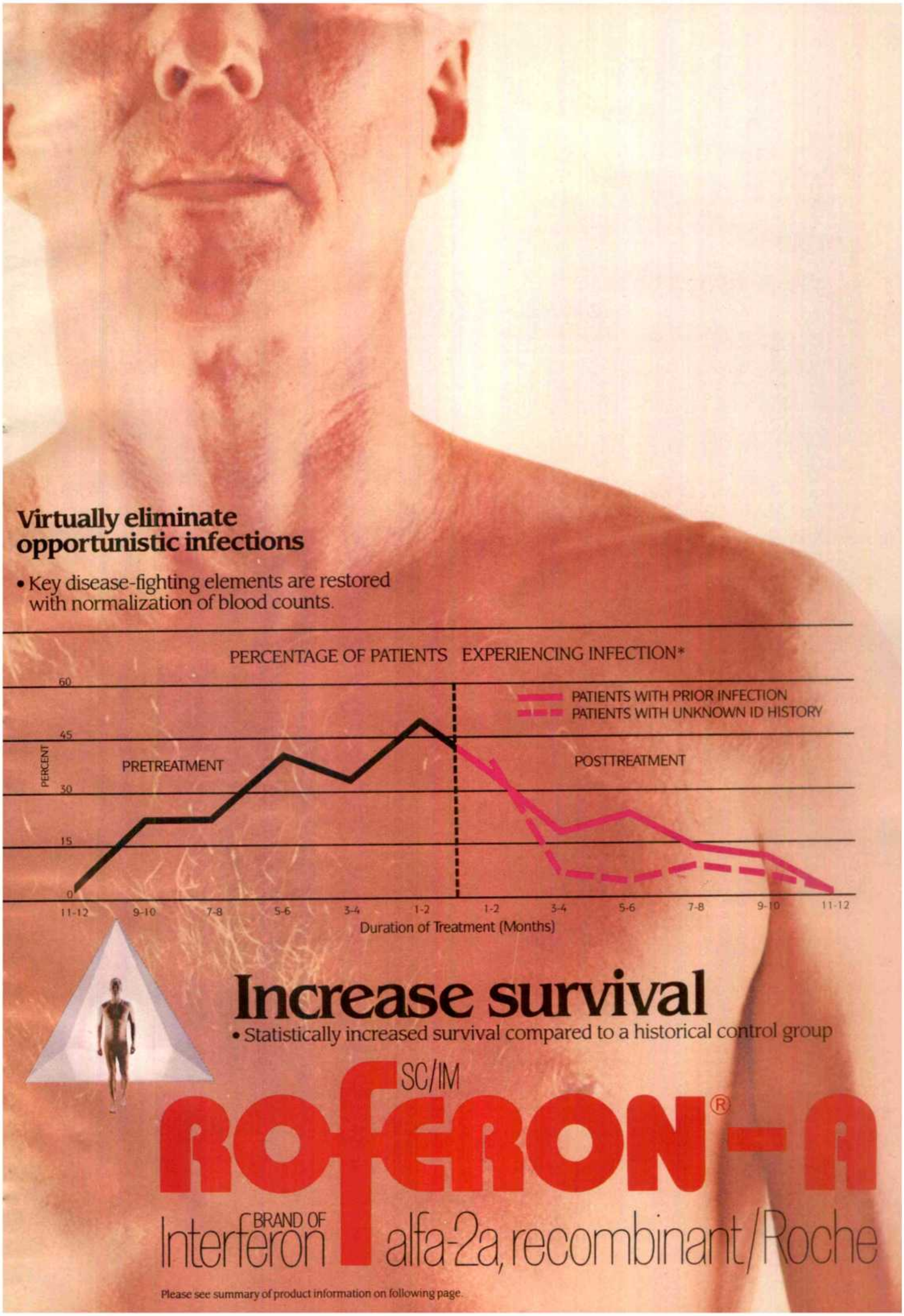

Virtually eliminate opportunistic infections

- Key disease-fighting elements are restored with normalization of blood counts.

Increase survival

- Statistically increased survival compared to a historical control group

SC/IM

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BRAND OF
Interferon alfa-2a, recombinant/Roche

Please see summary of product information on following page.

ROFERON®-A
Interferon alfa-2a, recombinant/Roche

Before prescribing, please consult complete product information, a summary of which follows:

CONTRAINDICATIONS: Hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Administer only under guidance of qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Use with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised CNS function.

Because of the possibility of severe or even fatal adverse reactions, inform patients of both benefits and risks.

Use with caution in patients with cardiac disease or *any* history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, fever, chills) frequently associated with administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred.

Exercise caution in administration to patients with myelosuppression.

CNS adverse reactions included decreased mental status, exaggerated CNS function, dizziness. More severe obtundation and coma rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of therapy. Careful periodic neuropsychiatric monitoring of all patients recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* When considering for chemotherapy, evaluate need and usefulness of the drug against risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, reduce dosage or discontinue drug and take appropriate corrective measures. Reinstitute therapy with caution and with adequate consideration of the further need for the drug and alertness to possible recurrence of toxicity.

The minimum effective dose in hairy cell leukemia not established.

Information for Patient: Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result. Inform patients regarding potential benefits and risks. If home use is desirable, give instructions on appropriate use, including review of the Patient Information Sheet. Patients should be well hydrated, especially during initial stages of treatment.

Laboratory Tests: Complete blood counts and liver function tests should be performed prior to therapy and at appropriate periods during therapy. Since responses are not generally observed for one to three months after treatment initiation, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase.

Patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility: Carcinogenesis: Roferon-A has not been tested for carcinogenic potential.

Mutagenesis: A. Internal studies. Ames test results revealed no evidence of mutagenicity. Human lymphocyte cultures were treated *in vitro* at noncytotoxic concentrations. No increase in chromosomal damage noted. B. Published studies. No published studies on mutagenic potential. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported. A chromosomal defect has been reported after the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder. Other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon. Protection from chromosomal aberrations produced by gamma rays observed in primary chick embryo fibroblasts.

Impairment of Fertility: Nonpregnant female rhesus monkeys treated at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. Monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Not fully evaluated.

PREGNANCY: Pregnancy Category C. Safe use in human pregnancy not established. Therefore, use during pregnancy only if potential benefit justifies potential risk to fetus. Primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Decreases in serum estradiol and progesterone concentrations reported in women treated with human leukocyte interferon. Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during therapy. Male fertility and teratologic evaluations have yielded no significant adverse effects.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants, a decision should be made whether to discontinue nursing or the drug, taking into account the importance of the drug to the mother.

Pediatric Use: Safety and effectiveness in children under 18 not established.

ADVERSE REACTIONS: The following data on adverse reactions are based on the SC or IM administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the U.S.

Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%), headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%), weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%), arthralgias (5%) were also observed. Rarely (<3%), CNS effects including decreased mental status, depression, visual disturbances, sleep disturbances, nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias, palpitations, were reported. Adverse experiences which occurred rarely and may have been related to underlying disease included epistaxis, bleeding gums, ecchymosis, petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis, inflammation at the site of injection, were also rarely observed.

Roferon-A has also been evaluated for the treatment of many other types of cancer under investigation in the U.S. These studies generally utilized higher doses (12 to 50 million IU/m^2). All of the above adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in the higher dosage group. Additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%), edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: *GI:* abdominal fullness, hypermotility, hepatitis; *CNS:* gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia, loss of libido; *Peripheral Nervous System:* muscle contractions; *Cardiovascular:* congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon, hot flashes; *Pulmonary:* bronchospasm, tachypnea; *Miscellaneous:* excessive salivation, flushing of skin.

Abnormal Laboratory Test Values: The percentages of abnormal laboratory test values seen in 1019 patients evaluated in overall safety trials (including hairy cell leukemia patients) are: *Hematologic:* leukopenia (69%), neutropenia (58%), thrombocytopenia (42%), decreased hemoglobin (6.3%), decreased hematocrit (12.5%); mean time to nadir: WBC—22 days, platelets—17 days. *Hepatic:* SGOT (78%), alkaline phosphatase (48%), LDH (47%), bilirubin (31%). *Renal/Urinary:* BUN (10%), serum creatinine (10%), uric acid (15%), proteinuria (25%). *Other Tests:* hypocalcemia (51%), elevated serum glucose (39%), elevated serum phosphorus (17%).

Neutralizing antibodies were detected in approximately 27% of all patients (3.4% for hairy cell leukemia patients). No clinical sequelae of their presence have been documented. Antibodies may occur spontaneously in certain clinical conditions.

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as an SC or IM injection. SC administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, 3 times/week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended. Treatment should be continued for 6 months before determining whether to discontinue therapy in nonresponding patients. The optimal duration of treatment has not been determined in responding patients. If severe reactions occur, modify dosage (50% reduction) or temporarily discontinue therapy until adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve.

HOW SUPPLIED: Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01). Roferon-A Injectable Solution: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09). Roferon-A Sterile Powder for Injection: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche, with accompanying diluent. Boxes of 1 (NDC 0004-1993-09). For reconstitution, storage and handling directions, see complete product information.

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Common side effects usually self-limiting and manageable

Initial side effects, such as fever, chills, myalgias, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug. Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function. Persistent fatigue is found to be less troublesome with *p.m.* or *h.s.* administration.

SC/IM fixed dosing simple enough for self-administration

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No mixing or reconstitution necessary

3 million IU vial (3 million IU/mL)

18 million IU vial (3 million IU/0.5 mL)

Sterile Powder for Injection

18 million IU vial (3 million IU/0.5 mL)

3 mL diluent for reconstitution

Must be refrigerated at temperatures of 36° to 46°F (2° to 8°C). At no time should Roferon-A Ready-to-Use Injectable Solution remain unrefrigerated for more than 24 hours. The Sterile Powder for Injection should not remain unrefrigerated for more than 48 hours. Once the powder is reconstituted, it should be refrigerated and must be used within 30 days. Do not freeze or shake.

SC/IM

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Interferon BRAND OF alfa-2a, recombinant/Roche

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Please see summary of product information on adjacent page.

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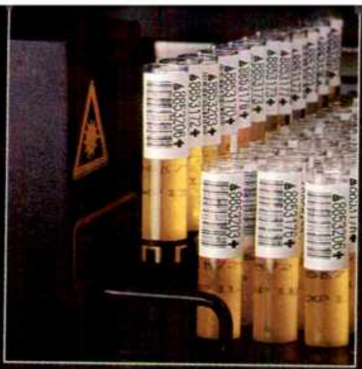

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New England Journal of Medicine reports

"[RETROVIR] AZT administration was therefore associated with a fourfold-to-sixfold reduction in the mortality rates in the study population at nine months."[1]

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A 90% survival rate was reported for AIDS and ARC patients treated with RETROVIR after one year— *v* 50% in a similar group of untreated patients[1,2]

Patients live better

Improves patients' general health and quality of life[1]

- Significantly reduces symptoms related to HIV infection
- Many patients can resume productive lives
- Reduces risk and severity of opportunistic infections
- Improves immune status

Improves patients' cognitive status[3,4]

- Produced statistically significant improvements in long-term memory, motor function, concentration, and information-processing ability
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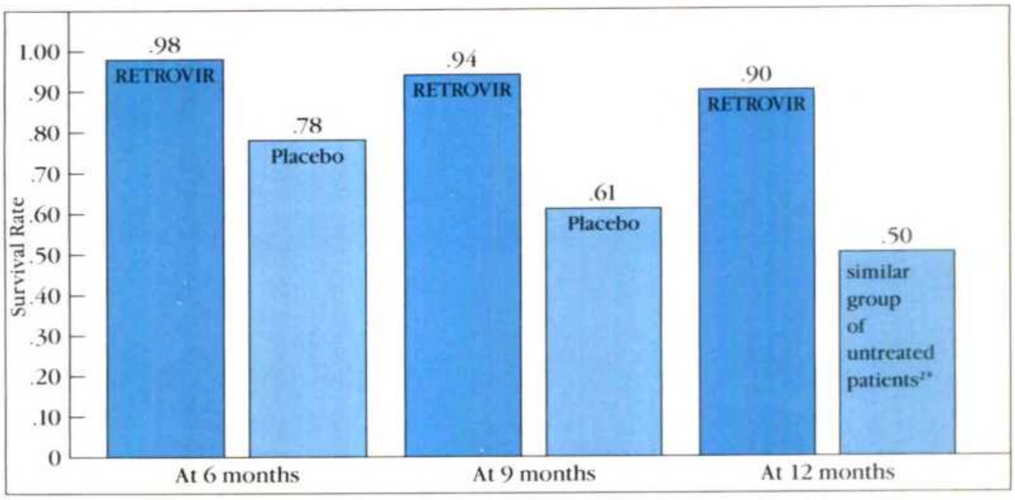
Helps patients live longer

Survival rates for AIDS/ARC patients combined at 6, 9, and 12 months[1,2]

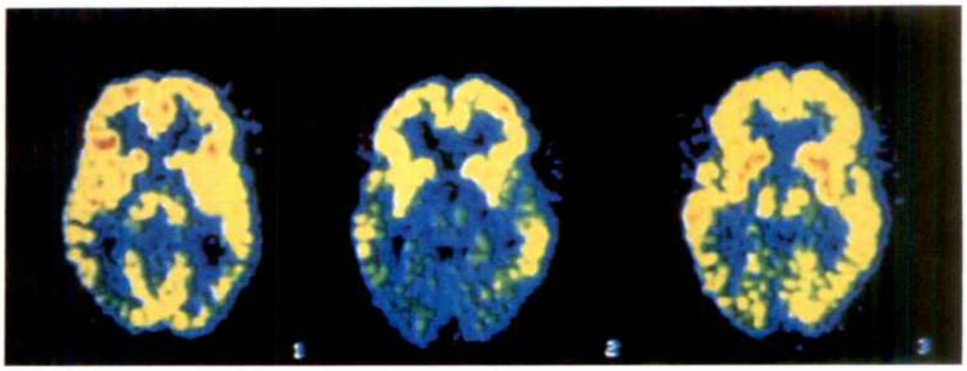


*Extrapolated from data of the Centers for Disease Control: *Morbidity and Mortality Weekly Report.*

Helps patients live better

Improvement following RETROVIR therapy demonstrated by positron emission tomography[3]

PET scans of normal control (1) and patient with HIV-related neuropathy and dementia before (2) and after (3) 13 weeks of RETROVIR therapy



Normal control (1)

Patient before RETROVIR therapy (2)

Patient following RETROVIR therapy (3)

A homogeneous pattern of glucose metabolism can be seen in the normal control (1) as compared with the heterogeneous pattern in the patient prior to RETROVIR therapy (2). After 13 weeks of treatment, the abnormal pattern has partly resolved (3).

Guidelines for successful management

- RETROVIR therapy is generally well tolerated by many patients, particularly those with higher T4 cell counts and more intact bone marrow function at the start of therapy
- Hematologic monitoring is recommended every two weeks; there is a possibility of bone marrow toxicity and subsequent RBC and WBC depression
- Concomitant use of acetaminophen with RETROVIR may potentiate granulocytopenia; other drugs that may interfere with RETROVIR metabolism should be avoided.

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (ZIDOVUDINE) Capsules

WARNING: THERAPY WITH RETROVIR (ZIDOVUDINE) IS OFTEN ASSOCIATED WITH HEMATOLOGIC TOXICITY INCLUDING GRANULOCYTOPENIA AND SEVERE ANEMIA REQUIRING TRANSFUSIONS (SEE WARNINGS).

IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OI'S) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than 200/mm^3 in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss ($\geq$ 15 lbs or $>$ 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 cells/mm^3 at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of $<$750/mm^3. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count $<$ 1000/mm^3 or hemoglobin $<$ 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 μg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/TK$^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 μg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 μg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 μg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 μg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine; 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly related to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table:

	Pretreatment CD4 (T4) Levels			
	≤200/mm^3		>200/mm^3	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia ($<$ 750/mm^3)	47%	10%	10%	3%
Anemia (Hgb $<$7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

		Pretreatment CD4 (T4) Levels			
		≤200/mm^3		>200/mm^3	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	>50%	55%	19%	40%	13%
Anemia	>25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.

Cardiovascular: vasodilation.

Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.

Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.

Nervous: anxiety, confusion, depression, emotional lability, nervousness, syncope, loss of mental acuity, vertigo.

Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.

Skin: acne, pruritus, urticaria.

Special senses: amblyopia, hearing loss, photophobia.

Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of $<$ 7.5 g/dL or reduction of $>$25% of baseline) and/or significant granulocytopenia (granulocyte count of $<$750/mm^3 or reduction of $>$50% from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.

References: **1.** Fischl MA, Richman DD, Grieco MH, et al: The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: A double-blind, placebo-controlled trial. *N Engl J Med* 1987;317:185-191. **2.** Acquired Immunodeficiency Syndrome (AIDS) Weekly Surveillance Report: United States Reported to CDC, July 9-12, 1987. **3.** Yarchoan R, Brouwers P, Spitzer AR, et al: Response of human-immunodeficiency-virus-associated neurological disease to 3′-azido-3′-deoxythymidine. *Lancet* 1987; 1:132-135. **4.** Data on file, Burroughs Wellcome Co.

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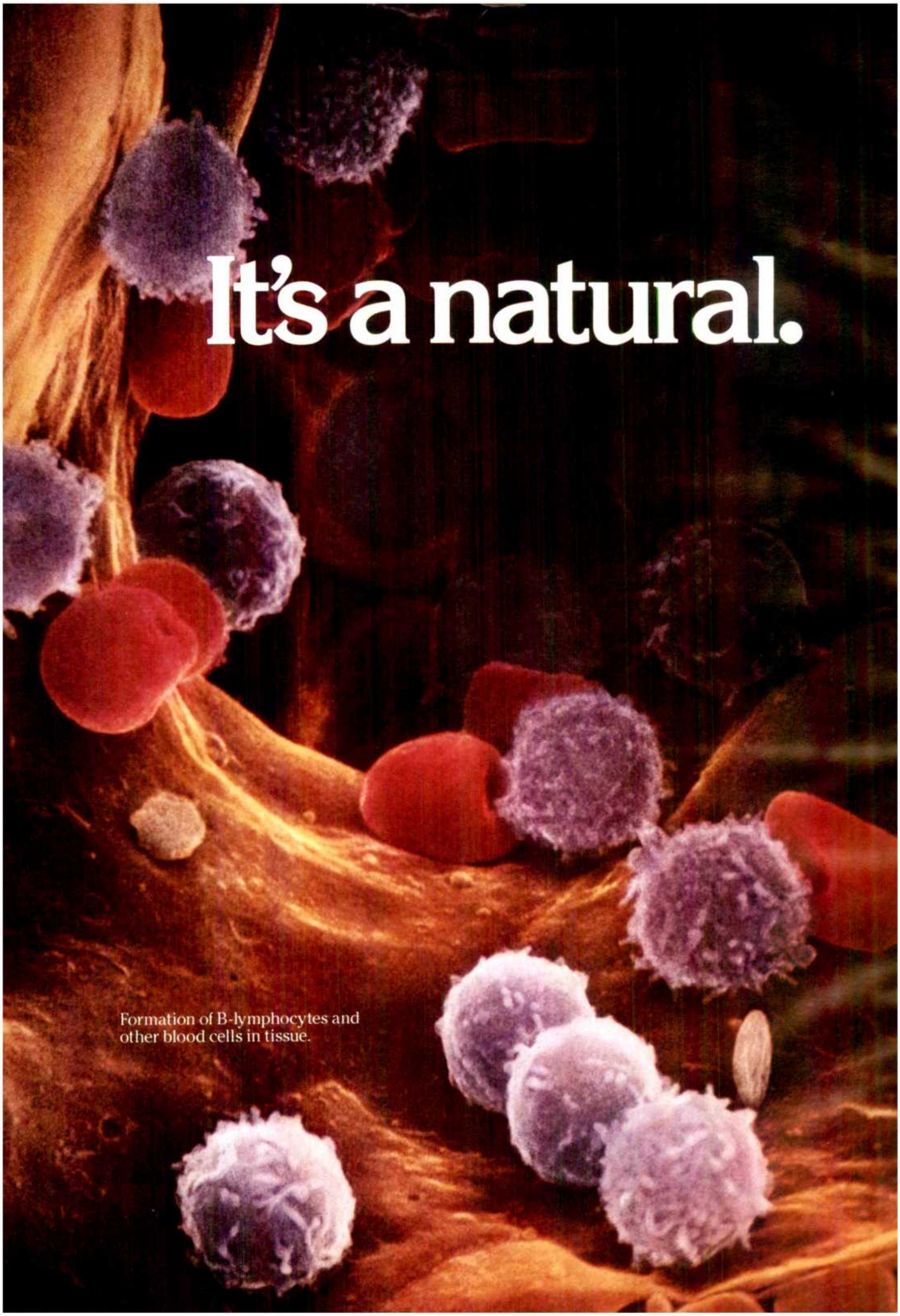

Formation of B-lymphocytes and other blood cells in tissue.

For ITP Patients

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

For patients with acute and chronic idiopathic thrombocytopenic purpura (ITP), maintaining normal platelet levels may be a life-threatening problem. They often need fast, aggressive therapy that will elevate platelet counts rapidly. They need Gamimune® N.

IGIV treatment with Gamimune® N may effect a therapeutic increase in platelets in both children and adults with ITP and, in some cases, allows the possibility of major surgery.[1] In one study,[1] 62.5% (10 of 16) adult patients and 83.3% (5 of 6) pediatric patients demonstrated a substantial response to an initial course of therapy with Gamimune® N.*†

Gamimune® N contains at least 98% gamma globulin and is the only ready-to-use liquid IGIV available. Cutter's patented fractionation method (based on Cohn-Oncley methods 6 and 9) assures Gamimune® N contains no heterologous proteins, with no detectable levels of protein aggregates or vasoactive enzymes.[2] And studies show *in vitro* inactivation of HIV (HTLV-III virus‡ during production of Gamimune® N.

Gamimune® N. Enhancing the natural process with fast, aggressive therapy for ITP in both children and adults.

---

*It is not possible to predict which patients with ITP will respond to therapy.

†In patients who respond to therapy, a rise in platelet count is generally rapid (within one to five days), transient (most often lasting from several days to several weeks), and should not be considered curative.

‡The Cohn-Oncley process used to produce Gamimune® N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III) and found to remove and/or inactivate the viral challenge. In addition, Gamimune® N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 reduction TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III).[3]

1. Gamimune® N Product License Application.
2. Lundblad JL, Mitra G, Sternberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(suppl 4): S382-S390.
3. Mitra G, Wong MF, Mozen M, et al. Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26:394-397.

See adjacent page for brief summary of prescribing information.

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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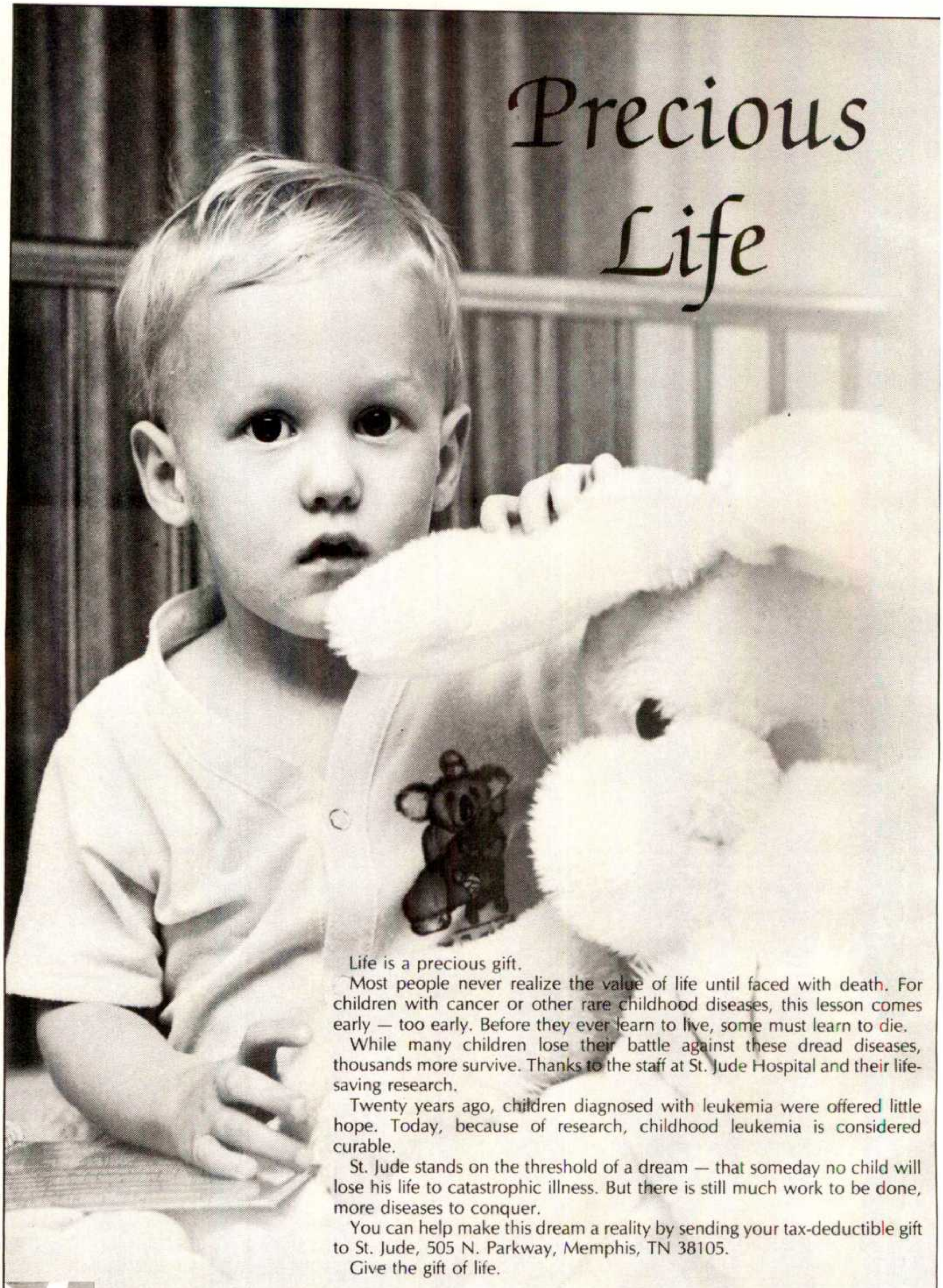

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Color-enhanced scanning electron micrograph of *Pneumocystis carinii.*

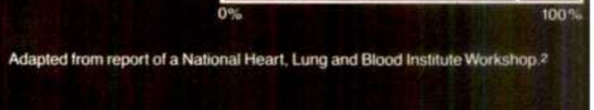
Drug of Choice in *PCP*

Bactrim I.V. is the drug of choice for treatment of *Pneumocystis carinii* pneumonia (*PCP*).[1] Please note, though, that AIDS patients may not tolerate or respond to treatment in the same way as non-AIDS patients.

The incidence of side effects—particularly rash, fever and leukopenia—is greatly increased in AIDS patients. Discontinue therapy at the first sign of skin rash or any adverse reaction.

High survival rate after first episodes of *PCP* in AIDS patients

In a multicenter evaluation of 185 AIDS patients who received trimethoprim plus sulfamethoxazole for their first episodes of *PCP,* 58% completed therapy with this combination alone. Among these patients, the rate of immediate survival was 82%.[2]



Adapted from report of a National Heart, Lung and Blood Institute Workshop.[2]

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

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References: 1. *Medical Letter 28*(706):9-16, Jan 31, 1986. **2.** Murray JF, *et al: N Engl J Med 310*(25):1682-1688, Jun 21, 1984.

Drug of choice in *PCP*.[1]

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

Before prescribing, please consult complete product information, a summary of which follows:

CONTRAINDICATIONS: Hypersensitivity to trimethoprim or sulfonamides; documented megaloblastic anemia due to folate deficiency; pregnancy at term and during the nursing period; infants less than two months of age.

WARNINGS: FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS.

BACTRIM SHOULD BE DISCONTINUED AT THE FIRST APPEARANCE OF SKIN RASH OR ANY SIGN OF ADVERSE REACTION. Clinical signs, such as rash, sore throat, fever, arthralgia, cough, shortness of breath, pallor, purpura or jaundice, may be early indications of serious reactions. In rare instances a skin rash may be followed by more severe reactions, such as Stevens-Johnson syndrome, toxic epidermal necrolysis, hepatic necrosis or sefious blood disorder. Perform complete blood counts frequently.

BACTRIM SHOULD NOT BE USED IN THE TREATMENT OF STREPTOCOCCAL PHARYNGITIS. Clinical studies have documented that patients with group A β-hemolytic streptococcal tonsillopharyngitis have a greater incidence of bacteriologic failure when treated with Bactrim than with penicillin.

Bactrim I.V. Infusion contains sodium metabisulfite, which may cause allergic-type reactions in susceptible persons, especially asthmatics or atopic nonasthmatic persons.

PRECAUTIONS:

General: Give with caution to patients with impaired renal or hepatic function, possible folate deficiency (*e.g.*, elderly, chronic alcoholics, patients on anticonvulsants, with malabsorption syndrome, or in malnutrition states) and severe allergies or bronchial asthma. In glucose-6-phosphate dehydrogenase deficient individuals, hemolysis may occur, frequently dose-related.

Local irritation and inflammation due to extravascular infiltration of the infusion have been observed. If these occur, discontinue infusion and restart at another site.

Use in the Elderly: May be increased risk of severe adverse reactions in elderly, particularly with complicating conditions, *e.g.*, impaired kidney and/or liver function, concomitant use of other drugs. Severe skin reactions, generalized bone marrow suppression (see WARNINGS and ADVERSE REACTIONS) or a specific decrease in platelets (with or without purpura) are most frequently reported severe adverse reactions in elderly. In those concurrently receiving certain diuretics, primarily thiazides, increased incidence of thrombocytopenia with purpura reported. Make appropriate dosage adjustments for patients with impaired kidney function (see DOSAGE AND ADMINISTRATION).

Use in the Treatment of Pneumocystis Carinii Pneumonitis in Patients with Acquired Immunodeficiency Syndrome (AIDS): Because of unique immune dysfunction, AIDS patients may not tolerate or respond to Bactrim in same manner as non-AIDS patients. Incidence of side effects, particularly rash, fever, leukopenia, with Bactrim in AIDS patients treated for *Pneumocystis carinii* pneumonitis reported to be greatly increased compared with incidence normally associated with Bactrim in non-AIDS patients.

Laboratory Tests: Perform appropriate culture and susceptibility studies before and throughout treatment. Complete blood counts should be done frequently; if a significant reduction in the count of any formed blood element is noted, discontinue Bactrim. Perform urinalyses with careful microscopic examination and renal function tests during therapy, particularly for patients with impaired renal function.

Drug Interactions: In elderly patients concurrently receiving certain diuretics, primarily thiazides, an increased incidence of thrombocytopenia with purpura has been reported. Bactrim may prolong the prothrombin time in patients who are receiving the anticoagulant warfarin. Keep this in mind when Bactrim is given to patients already on anticoagulant therapy and reassess coagulation time. Bactrim may inhibit the hepatic metabolism of phenytoin. Given at a common clinical dosage, it increased the phenytoin half-life by 39% and decreased the phenytoin metabolic clearance rate by 27%. When giving these drugs concurrently, be alert for possible excessive phenytoin effect. Sulfonamides can displace methotrexate from plasma protein binding sites, thus increasing free methotrexate concentrations.

Drug/Laboratory Test Interactions: Bactrim, specifically the trimethoprim component, can interfere with a serum methotrexate assay as determined by the competitive binding protein technique (CBPA) when a bacterial dihydrofolate reductase is used as the binding protein. No interference occurs if methotrexate is measured by a radioimmunoassay (RIA). The presence of trimethoprim and sulfamethoxazole may also interfere with the Jaffé alkaline picrate reaction assay for creatinine, resulting in overestimations of about 10% in the range of normal values.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Carcinogenesis: Long-term studies in animals to evaluate carcinogenic potential not conducted with Bactrim I.V. Infusion. *Mutagenesis:* Bacterial mutagenic studies not performed with sulfamethoxazole and trimethoprim in combination. Trimethoprim demonstrated to be nonmutagenic in the Ames assay. No chromosomal damage observed in human leukocytes cultured *in vitro* with sulfamethoxazole and trimethoprim alone or in combination; concentrations used exceeded blood levels of these compounds following therapy with Bactrim. Observations of leukocytes obtained from patients treated with Bactrim revealed no chromosomal abnormalities. *Impairment of Fertility:* Bactrim I.V. Infusion not studied in animals for evidence of impairment of fertility. Studies in rats at oral dosages as high as 70 mg/kg trimethoprim plus 350 mg/kg sulfamethoxazole daily showed no adverse effects on fertility or general reproductive performance.

BACTRIM™ I.V. Infusion (trimethoprim and sulfamethoxazole/Roche)

Pregnancy: Teratogenic Effects: Pregnancy Category C. Trimethoprim and sulfamethoxazole may interfere with folic acid metabolism; use during pregnancy only if potential benefit justifies potential risk to fetus. Nonteratogenic Effects: See CONTRAINDICATIONS section.

Nursing Mothers: See CONTRAINDICATIONS section.

Pediatric Use: Not recommended for infants under two months (see CONTRAINDICATIONS section).

ADVERSE REACTIONS: Most common are gastrointestinal disturbances (nausea, vomiting, anorexia) and allergic skin reactions (such as rash and urticaria). **FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS (SEE WARNINGS SECTION).** Local reaction, pain and slight irritation on I.V. administration infrequent. Thrombophlebitis rarely observed.

Hematologic: Agranulocytosis, aplastic anemia, thrombocytopenia, leukopenia, neutropenia, hemolytic anemia, megaloblastic anemia, hypoprothrombinemia, methemoglobinemia, eosinophilia. *Allergic Reactions:* Stevens-Johnson syndrome, toxic epidermal necrolysis, anaphylaxis, allergic myocarditis, erythema multiforme, exfoliative dermatitis, angioedema, drug fever, chills, Henoch-Schoenlein purpura, serum sickness-like syndrome, generalized allergic reactions, generalized skin eruptions, conjunctival and scleral injection, photosensitivity, pruritus, urticaria and rash. Periarteritis nodosa and systemic lupus erythematosus have been reported. *Gastrointestinal:* Hepatitis (including cholestatic jaundice and hepatic necrosis), elevation of serum transaminase and bilirubin, pseudomembranous enterocolitis, pancreatitis, stomatitis, glossitis, nausea, emesis, abdominal pain, diarrhea, anorexia. *Genitourinary:* Renal failure, interstitial nephritis, BUN and serum creatinine elevation, toxic nephrosis with oliguria and anuria, crystalluria. *Neurologic:* Aseptic meningitis, convulsions, peripheral neuritis, ataxia, vertigo, tinnitus, headache. *Psychiatric:* Hallucinations, depression, apathy, nervousness. *Endocrine:* Sulfonamides bear certain chemical similarities to some goitrogens, diuretics (acetazolamide and the thiazides) and oral hypoglycemic agents; cross-sensitivity may exist. Diuresis and hypoglycemia have occurred rarely in patients receiving sulfonamides. *Musculoskeletal:* Arthralgia, myalgia. *Respiratory:* Pulmonary infiltrates. *Miscellaneous:* Weakness, fatigue, insomnia.

DOSAGE AND ADMINISTRATION: CONTRAINDICATED IN INFANTS LESS THAN TWO MONTHS OF AGE. CAUTION—BACTRIM I.V. INFUSION MUST BE DILUTED IN 5% DEXTROSE IN WATER SOLUTION PRIOR TO ADMINISTRATION. DO NOT MIX WITH OTHER DRUGS OR SOLUTIONS. RAPID INFUSION OR BOLUS INJECTION MUST BE AVOIDED.

DOSAGE *(Children and Adults):*

PNEUMOCYSTIS CARINII PNEUMONITIS: Total daily dose is 15 to 20 mg/kg (based on the trimethoprim component) given in three or four equally divided doses every 6 to 8 hours for up to 14 days. One investigator noted that a total daily dose of 10 to 15 mg/kg was sufficient in 10 adults with normal renal function.

SEVERE URINARY TRACT INFECTIONS AND SHIGELLOSIS: Total daily dose is 8 to 10 mg/kg (based on the trimethoprim component) given in two to four equally divided doses every 6, 8 or 12 hours for up to 14 days for severe urinary tract infections and 5 days for shigellosis. Maximum recommended daily dose is 60 ml. *Renal Impaired:* Creatinine clearance above 30 ml/min, give usual dosage; 15-30 ml/min, give one-half the usual regimen; below 15 ml/min, use not recommended.

Method of Preparation: Bactrim I.V. Infusion must be diluted. EACH 5 ML SHOULD BE ADDED TO 125 ML OF 5% DEXTROSE IN WATER. After diluting do not refrigerate; use within 6 hours. If a dilution of 5 ml/100 ml of 5% dextrose in water is desired, use within 4 hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

Multidose Vials: After initial entry into the vial, use remaining contents within 48 hours. The following infusion systems are satisfactory: unit-dose glass containers; unit-dose polyvinyl chloride and polyolefin containers.

Dilution: ADD EACH 5 ML OF BACTRIM I.V. INFUSION TO 125 ML OF 5% DEXTROSE IN WATER.

NOTE: *When fluid restriction is desirable*, add each 5 ml to 75 ml of 5% dextrose in water; mix just prior to use and administer within two hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

DO NOT MIX BACTRIM I.V. INFUSION—5% DEXTROSE IN WATER WITH DRUGS OR SOLUTIONS IN THE SAME CONTAINER.

ADMINISTRATION: Administer by I.V. infusion over 60 to 90 minutes. Avoid rapid infusion or bolus injection. Do not give intramuscularly.

HOW SUPPLIED: 5-ml ampuls, 5-ml vials and 10-ml vials—boxes of 10; 30-ml and 50-ml multidose vials—boxes of 1. Each 5 ml contains 80 mg trimethoprim (16 mg/ml) and 400 mg sulfamethoxazole (80 mg/ml) for infusion with 5% dextrose in water.

STORE AT ROOM TEMPERATURE (15°-30°C or 59°-86°F). DO NOT REFRIGERATE.

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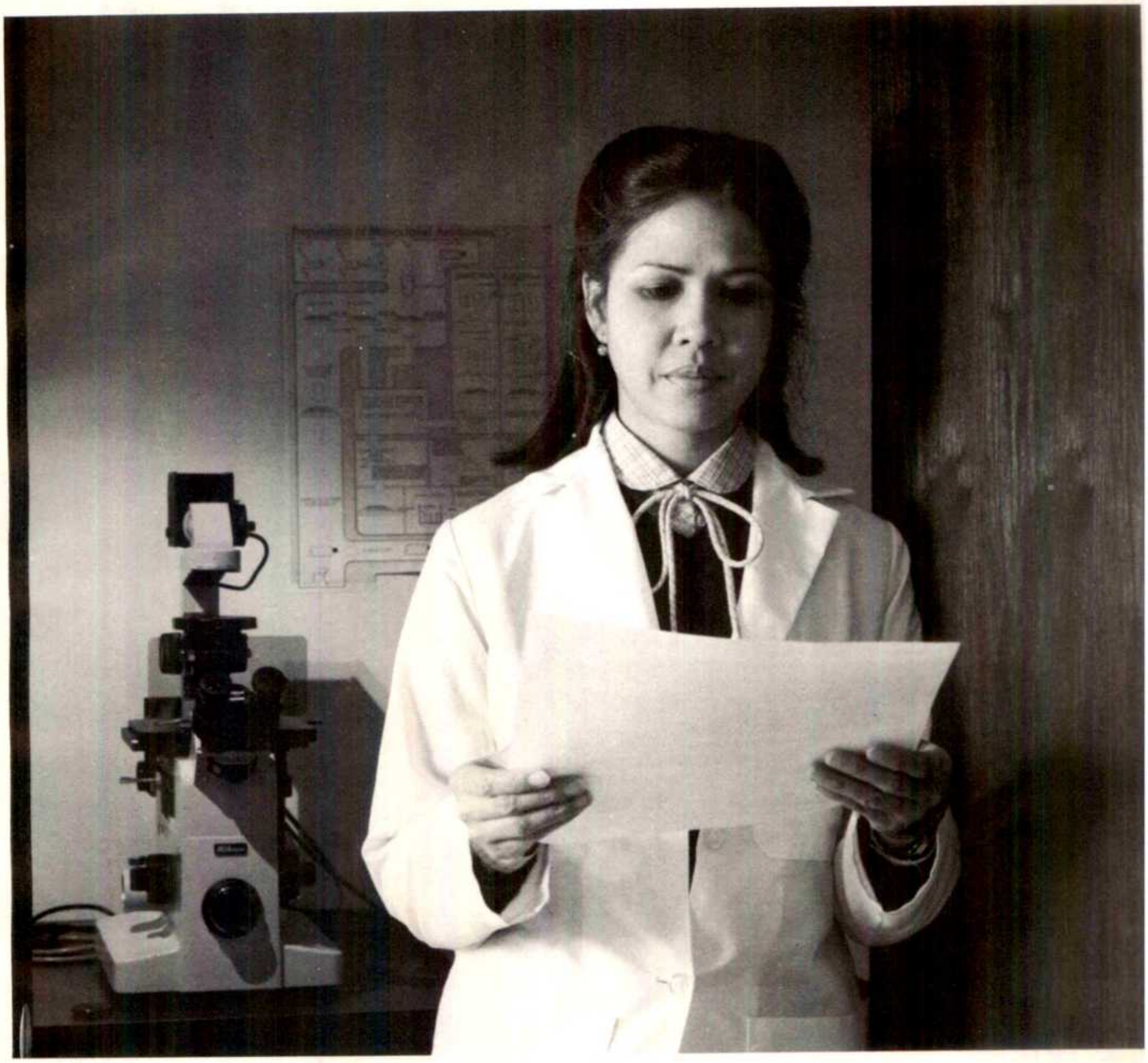

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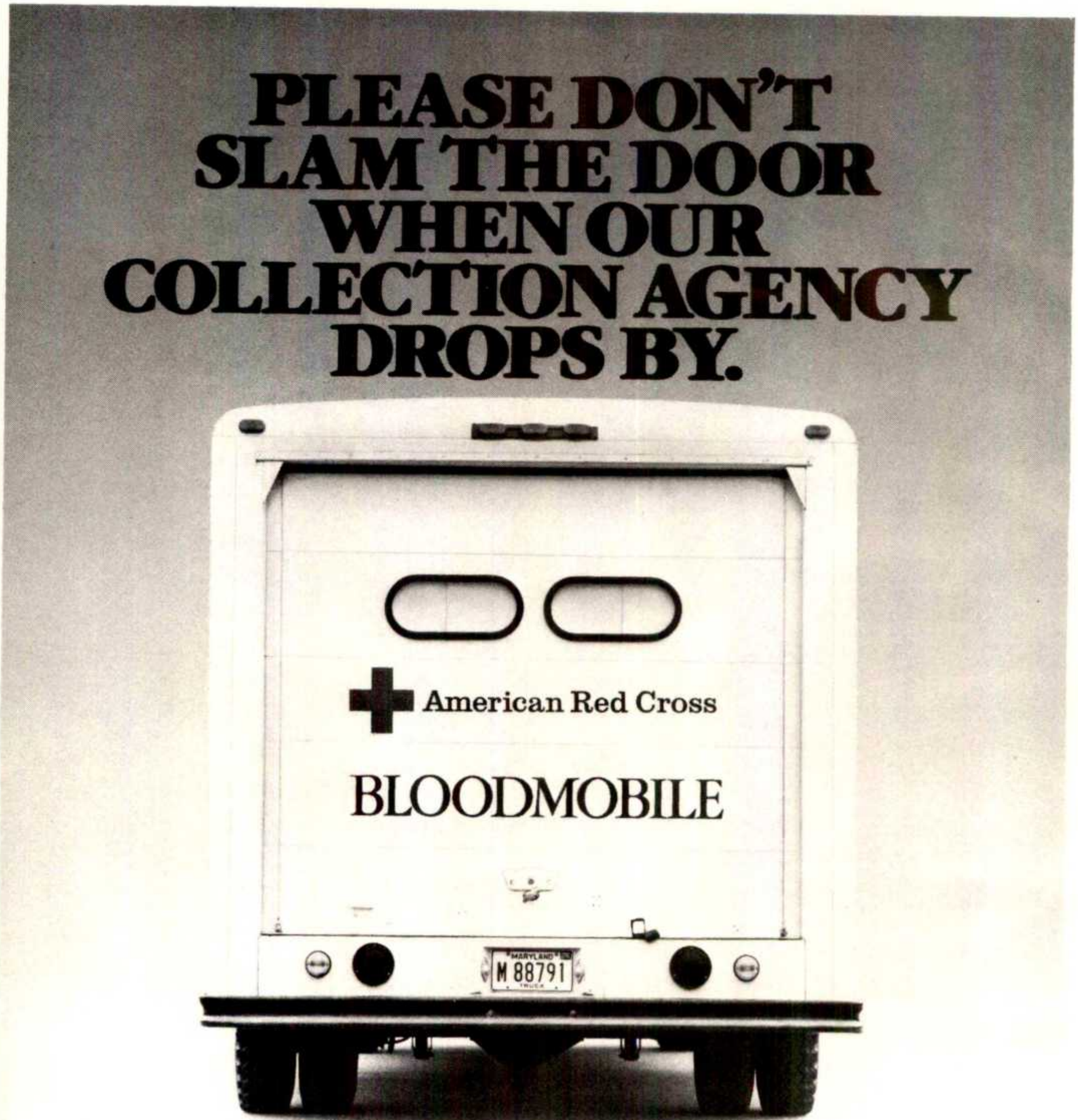
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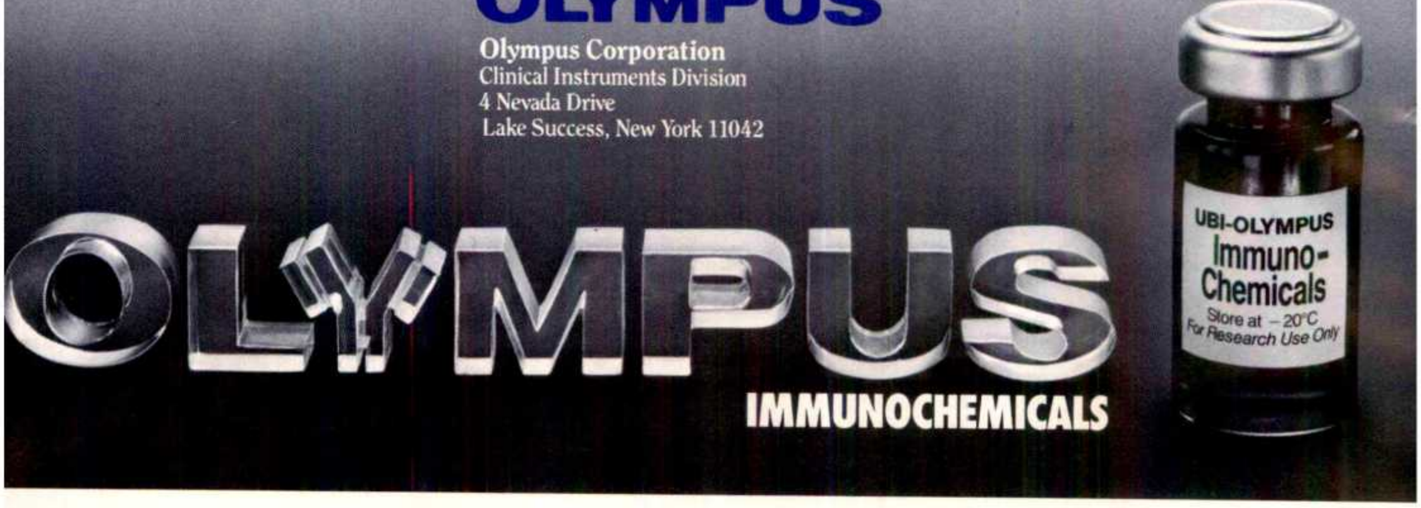

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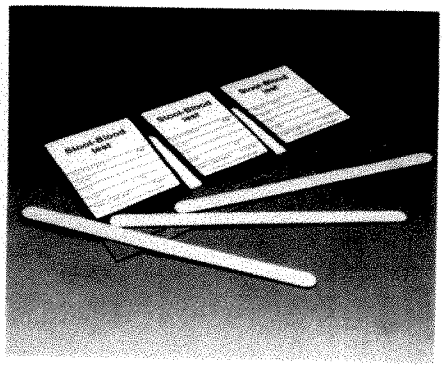

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Two days before the test, you begin a diet you might enjoy all the time. Plenty of fresh vegetables raw or cooked, especially corn, spinach and lettuce. Lots of plums, grapes, apples and prunes, moderate amounts of peanuts and popcorn. No red meat, turnips or horseradish. Do's and don't's are listed in the kit.

The presence of hidden blood usually indicates some problem in the stomach or bowel, not necessarily cancer. Positive tests must be followed by further testing to find out what the problem is.

Other tests for colorectal cancer you should talk to your doctor about: Digital rectal exam (after age 40); the procto test (after age 50). It is important to report any personal or family history of intestinal polyps or ulcerative colitis, and any change in your bowel habits, which could be a cancer warning signal.

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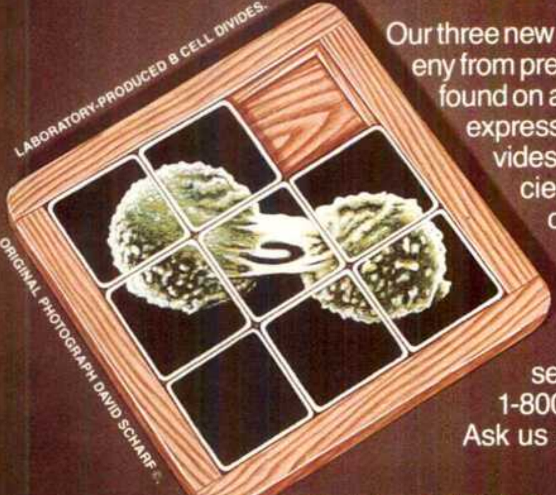

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Please see next page for
brief summary and references.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane* be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents.

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of the lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain $FI\ O_2$ at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban*. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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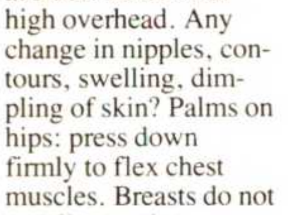

2. In front of a mirror.
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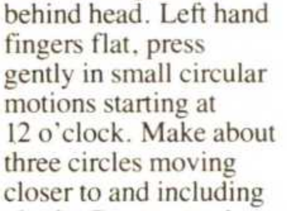

3. Lying down.
Pillow under right shoulder, right hand behind head. Left hand fingers flat, press gently in small circular motions starting at 12 o'clock. Make about three circles moving closer to and including nipple. Reverse and repeat on left.

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REVIEW ARTICLE

The HL-60 Promyelocytic Leukemia Cell Line: Proliferation, Differentiation, and Cellular Oncogene Expression

By Steven J. Collins

The HL-60 cell line, derived from a single patient with acute promyelocytic leukemia, provides a unique in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage.

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ALTHOUGH LEUKEMIC cells from patients with acute myeloid leukemia display a seemingly unlimited proliferative capacity in vivo, their life span in liquid suspension culture is usually limited to a few cell divisions. One possible explanation for such limited in vitro proliferation is that certain growth factors sustaining leukemic cell division in the patient are absent from the in vitro culture environment. About 10 years ago, investigators in Dr R. C. Gallo's laboratory at the National Institutes of Health (NIH), in an attempt to identify such leukemia-specific growth factors, cultured fresh human leukemic cells with conditioned media derived from cultures of both human embryonic cells and mitogen-stimulated lymphocytes.[1] Although the postulated leukemia-specific growth factor was never reproducibly identified, this experimental approach proved fruitful in a number of unexpected ways. The T cell mitogen interleukin 2 (TCGF) was first identified,[2] and a factor-independent immortal cell line (HL-60) with distinct myeloid characteristics was developed.[3,4] The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, can be induced to differentiate in vitro to a number of different cell types, and studies with this leukemic cell line have proved invaluable in a variety of different areas. These include: (a) providing insight into the control mechanisms of normal granulocyte/monocyte/macrophage differentiation; (b) suggesting new therapeutic approaches for patients with leukemia; (c) providing a ready source of cDNA for cloning of important granulocyte and monocyte enzymes and monokines; (d) providing insight into possible mechanisms of rapid regulation of gene transcription; and (e) serving as an invaluable model for studying specific cellular oncogene expression in relationship to particular hematopoietic differentiative lineages. This review attempts to summarize the important characteristics of this unique cell line, with particular emphasis on HL-60 proliferation, differentiation, and cellular oncogene expression.

GROWTH AND PROLIFERATION

HL-60 promyelocytic leukemia cells continuously proliferate in suspension culture with a doubling time of ~36 to 48 hours. This characteristic alone makes HL-60 unusual among human myeloid leukemias. Most fresh myeloid leukemia cells when cultured in liquid suspension undergo a limited number of cell divisions prior to growth arrest and cell death, and relatively few human leukemic cell lines displaying distinct myeloid or myelomonocytic characteristics have been established. What are the characteristics of HL-60 cells that make them adapted to such continuous growth in liquid suspension? HL-60 cell surface expression of transferrin and insulin receptors appears critical to their proliferative capacity. HL-60 cells can proliferate in serum-free nutrient media (eg, RPMI 1640) provided that it is supplemented with transferrin and insulin.[5] The requirement for insulin and transferrin is absolute, as HL-60 proliferation immediately ceases if either of these compounds is removed from the serum-free culture media.[5] Transferrin requirement by HL-60 as well as by other cultured cells[6] indicates that an active iron transport system is necessary for cell proliferation. HL-60 cells express the transferrin receptor on the cell surface, and this receptor expression markedly decreases as the cells are induced to differentiate terminally.[7,8] Moreover, monoclonal antibodies to the transferrin receptor inhibit HL-60 cell proliferation.[9] Similarly, insulin receptors are also displayed by HL-60 cells, and insulin receptor expression decreases with granulocytic differentiation of HL-60.[10] The presence of the insulin and transferrin receptors on HL-60 cells cannot, however, in itself explain the unusual in vitro proliferative capacity of this cell line since a number of fresh leukemia cells also display these

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receptors but nevertheless are unable to grow continuously in culture.

The in vitro proliferation of normal myeloid progenitor cells is stimulated by the colony-stimulating factor (CSF) family of compounds. Certain CSFs also stimulate the proliferation of HL-60 cells.[11-13] HL-60 cells have been postulated to produce certain CSF-like compounds that act as autostimulators of HL-60 growth, and several compounds exhibiting such "autocrine" activity have been partially purified from HL-60–conditioned media. Brennan et al described a 13,000 mol wt compound produced by HL-60 cells which stimulated HL-60 cell growth but which exhibited no CSF activity on normal mouse bone marrow cells.[14] Perkins et al described a 25,000 mol wt HL-60 product with similar HL-60 autostimulatory activity. In addition, this compound exhibits CSF activity on normal bone marrow and shares antigenic determinants with certain CSFs.[15] These HL-60 autostimulatory products have not been molecularly cloned, and it is presently unclear what their exact relationship is to each other or to the normal CSF molecules. One of the chromosome 5 GM-CSF alleles appears to be partially deleted in HL-60 cells,[16] but there is no evidence that this gene is expressed in HL-60 cells, and the effect, if any, of this genomic rearrangement on the proliferation of HL-60 is presently unknown.

HL-60 cells also exhibit genetic abnormalities in specific cellular oncogenes (see below), and these abnormalities most likely also play a role in the unusual ability of these myeloid leukemic cells to proliferate continuously in vitro.

DIFFERENTIATION

The characteristic of HL-60 cells that has attracted the most research interest is their capacity to differentiate in vitro to a variety of different cell types of the myelomonocytic lineage. Various agents induce HL-60 cells to differentiate to four general types of cells: (a) granulocytes, (b) monocytes, (c) macrophagelike cells, and (d) eosinophils. These designations are somewhat arbitrary, and certain HL-60–inducing agents give rise to cells with characteristics that overlap these categories. Moreover, as emphasized later, the induced HL-60 cells are usually deficient in certain characteristics of their normal cell counterpart. Nevertheless, these categories provide a convenient outline for discussing the numerous and complex phenotypic changes induced in this leukemic cell line by a wide variety of agents. These categories are discussed separately below.

Granulocytes. The HL-60 cells are predominantly promyelocytes but in most cell cultures, particularly passage 60 or less, ~5% of the cells exhibit spontaneous differentiation to morphologically mature cells including myelocytes, metamyelocytes, banded and segmented neutrophils.[3] The addition to these cultures of specific inducing agents, including polar-planer compounds such as dimethyl sulfoxide DMSO,[17] or other agents such as retinoic acid[18] markedly increases this spontaneous differentiation, with most cells acquiring morphological, functional, enzymatic, and surface membrane antigen characteristics of mature granulocytes (Table 1). A variety of different compounds induces such granulocytic differentiation of HL-60 cells (Table 2). Many of these compounds also induce erythroid differentiation in Friend mouse erythroleukemia cells.[19,20] By several criteria, this granulocytic differentiation of HL-60 cells is somewhat incomplete and defective. For example, most granulocyte-induced cultures consist predominantly of metamyelocytes and banded neutrophils rather than fully differentiated multilobulated polys. In addition, the granulocyte-induced HL-60 cells lack lactoferrin, suggesting that they are deficient in secondary granules.[21,22] The LDH isoenzyme profile of granulocyte-induced HL-60 differs quantitatively from the LDH isoenzymes of normal granulocytes consistent with incomplete differentiation.[23] Deficiencies have been described in the myeloperoxidase/peroxide/halide killing sys-

Table 1. Phenotypic Characteristics of Induced HL-60 Cells

Characteristic	Uninduced	Granulocyte	Monocyte	Macrophagelike	Eosinophil
Myeloperoxidase	+[3,4]	↓[22,147]	↓[22,42]	↓[61]	+[96,97]
Eosinophil peroxidase	−[97]				+[96,97]
ASD chloroacetate esterase	+[4]	+[4]	±[42,51]	↓[61]	+[97]
Nonspecific esterase	−[4,61]	−[61]	+[51]	+[61]	−[97]
Acid phosphatase	+[148]	↑[148]		↑↑[148]	+[97]
Biebrich scarlet	−[96]	−[96]		±[96]	+[96]
Plastic adherence	−[61,155]	−[61,155]	−[43,155]	+[61]	
Chemotaxis	−[74,150]	+[74,150]	+[149]	−[152]	
Chemotactic receptors	+[150,151]	↑[150,151]	↑[56,149]	−[25]	
Complement receptors	+[47,74]	↑[47,74]	↑[47]	↑[47]	
F_c receptors	+[47]	↑[47]	↑[56]	↑[56,152]	
Lysozyme	+[4,61]	↑[61]		↑↑[61]	
NBT reduction	−[74]	+[74]	+[52]	−[25,66]	
Phagocytosis	−[17,47,74]	+[17,47,74]	+[47]	+[47,61]	
Microbicidal	−[21]	+[21]		+[152]	
Monocyte/macrophage surface antigens	±[42,154]	±[44,65,154]	+[42-44,154]	+[42,65]	
Granulocyte surface antigens	±[65,153]	+[65,153]	−[65]	−[65]	
Insulin receptors	+[10]	↓[10]	↑[10]		
Transferrin receptors	+[7,8]	↓[7,8]		↓[87]	

Arrows indicate a quantitative increase (↑) or decrease (↓) from uninduced HL-60 levels. Blank spaces correspond to characteristics absent from the literature, (+) indicates present, (−) indicates absent, ± indicates discrepant or equivocal literature findings.

Table 2. Inducers of Differentiation of HL-60 Cells

	Induced Cell Type			
	Granulocyte	Monocyte	Macrophagelike	Eosinophil
Inducing agents	Polar-planer compounds[17]	Vitamin D_3[47-50]	Phorbol esters (TPA)[61,62]	Alkaline media[97]
	Retinoic acid[18]	Na Butyrate[155]	Teleocidin[161,162]	Butyric acid[98]
	Actinomycin D[20]	Lymphocyte-conditioned media[51]		GM-CSF[5]
	Hypoxanthine[20]	DIF[53]		
	Antithymocyte globulin[156]	IFN-γ[45,54-56]		
	Tunicamycin[157]	Tumor necrosis factor[59]		
	6-thioguanine[20]	Ara C[159]		
	L-ethionine[158]	Ara A[160]		

tem in HL-60 granulocytes.[24] Unlike other granulocyte inducers, retinoic acid treated HL-60 granulocytes do not display an increase in chemotactic peptide receptors.[25]

Despite these deficiencies, most of the granulocyte-inducing agents induce terminal differentiation of HL-60 cells; that is, the induced cells are no longer capable of proliferation. This together with the functional maturation of these cells, which enables them to engage in the killing of microorganisms (Table 1), suggests that these granulocyte inducers, particularly retinoic acid, may be of therapeutic benefit in patients with myelogenous leukemia. Unfortunately, HL-60 cells appear to be quite exceptional among acute nonlymphocytic leukemias (ANL) in their response to granulocyte differentiation inducers. Indeed, most fresh leukemic cells in short-term culture in the presence of granulocyte-inducing agents do not exhibit differentiation.[26,27] Nevertheless, a subset of ANL patients exists, primarily those classified as acute promyelocytic leukemia, whose leukemic cells clearly exhibit induced granulocytic differentiation in vitro.[26] Moreover, there are several anecdotal reports of patients with promyelocytic leukemia and with myelodysplastic syndrome who have apparently benefited from systemic therapy with retinoic acid.[28-32]

The mechanism of action of the HL-60 granulocyte inducers is unknown. At least a 12-hour exposure to DMSO or retinoic acid is necessary before any differentiation occurs and continuous exposure to these compounds generally is required to induce maximum differentiation.[18,33] Although retinoic acid is believed to exert its biologic effect in many cells by interacting with a specific cytoplasmic retinoic-acid binding protein (RABP),[34,35] our own observations and those of other researchers[36] indicate that HL-60 cells lack such a binding protein. Evidence that retinoic acid-induced HL-60 differentiation is initiated at the cell surface is provided by the observation that granulocytic differentiation occurs in HL-60 cells when cultured with retinoic acid immobilized on a solid substrate.[37] HL-60 cell lines resistant to the differentiation-inducing effects of retinoic acid exhibit an apparent high-mol-wt acidic surface membrane glycoprotein that is absent from the wild-type HL-60 cells.[38,39] This aberrant surface membrane glycoprotein is hyposialyated, but its relationship to the retinoic acid resistance is presently uncertain.[40]

Monocytes. Different compounds induce HL-60 to differentiate to cells exhibiting distinct "monocytic" characteristics. These cells morphologically can be confused with immature HL-60 granulocytes, and they share many important functional characteristics with HL-60 cells treated with granulocytic inducers (Table 1). They can be readily distinguished from these latter cells, however by different markers, including positive staining for the monocyte/macrophage specific enzyme α-napthyl acetate esterase (nonspecific esterase-NSE),[41] by the presence of monocyte specific cell membrane antigens,[42-44] and by their capacity for mediating antibody-dependent cytoxicity (ADCC).[44-46]

A variety of different naturally occurring compounds induces monocytic differentiation of HL-60. These include vitamin D_3 as well as various products found in mitogen-stimulated lymphocyte-conditioned media (LCM).[47-51] 1,25 dihydroxyvitamin D_3 induces monocytic differentiation of HL-60 cells at concentrations as low as 10^{-10} mol/L[47-50] and specific high-affinity cytosol receptors for vitamin D_3 have been observed in HL-60.[48] This compound also induced monocyte/macrophage differentiation in normal human bone marrow cells.[49] Vitamin D_3 could have therapeutic potential but, as with retinoic acid, HL-60 cells appear to be unusual among ANL cells in their differentiative response to this agent. Other naturally occurring compounds of interest that induce monocytic differentiation of HL-60 cells include various products produced by mitogen-stimulated mononuclear cell cultures.[51] An HL-60 monocyte-inducing compound with mol wt of ~40,000 termed differentiation-inducing factor (DIF) has been isolated from LCM and from CM from the T cell leukemia cell line HUT-102.[52,53] Another mitogen-stimulated lymphocyte product, γ-interferon, (IFN-γ) induces monocytic differentiation of HL-60,[45,54-56] whereas IFN-α and IFN-β alone do not exhibit this inducing capacity. Synergism among different naturally occurring compounds has been demonstrated in the induction of monocytic differentiation of HL-60. For example, retinoic acid in relatively small concentrations synergistically acts with DIF,[57] IFN-γ[46] and IFN-α[46,58] to induce monocytic differentiation of HL-60. This latter finding is interesting since retinoic acid by itself is a granulocytic inducer of HL-60. In addition, lymphotoxin and tumor necrosis factor (cachectin), which are also products of mitogen-stimulated mononuclear cells, also act in synergy to promote IFN-γ–induced differentiation of HL-60.[59]

Therapeutically, monocytic differentiation of HL-60, unlike granulocytic differentiation, is not necessarily accompanied by the loss of proliferative capacity of the induced cells (ie, monocytic HL-60 differentiation is not necessarily terminal differentiation). For example, recombinant IFN-γ alone as well as IFN-γ plus retinoic acid induces monocytic characteristics of HL-60 cells, but they have little effect on inhibiting HL-60 cell proliferation.[45,46] Moreover, Elias et al identified a factor in mitogen-stimulated LCM that markedly enhances thymidine incorporation of HL-60 cells.[60] An early stimulatory effect of tumor necrosis factor on HL-60 thymidine incorporation has also been observed.[59] Thus, monocytic differentiation of HL-60 may in certain cases be preceded by enhanced cell proliferation, and HL-60 cells induced to acquire particular monocytic characteristics have not necessarily lost the capacity to proliferate.

Macrophagelike. The primary inducer of macrophagelike HL-60 cells is the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). This TPA-induced macrophagelike phenotype is frequently combined with the "monocytic" phenotype in discussions of HL-60 differentiation; indeed, these induced cell types both share many of the same enzymatic, histochemical, and functional characteristics (Table 1). Several important phenotypic characteristics clearly distinguish the HL-60 macrophagelike differentiation from HL-60 monocytic differentiation, however. First, TPA-treated HL-60 cells are markedly more adherent than HL-60 monocytes. TPA-treated cells exhibit intense adherence to plastic with prominent pseudopodia formation and also markedly adhere to each other.[61,62] Second, several specific surface antigens present on normal and HL-60 monocytes are not induced in TPA-treated HL-60 cells.[42,43,50,65] Third, no induction of chemotactic peptide receptors or of NBT reduction activity occurs in TPA-treated HL-60,[25,66] whereas these characteristics are readily demonstrated in HL-60 monocytes.[52,56] In contrast to monocytic induction, TPA induction of HL-60 is associated with a rapid profound loss in proliferative capacity. Few HL-60 cells are able to enter S-phase after exposure to TPA.[61,62] Moreover, TPA-induced HL-60 differentiation can occur in the absence of cell division or new DNA synthesis.[63,64] Fifth, in contrast to the HL-60 granulocyte and monocyte inducers, prolonged exposure to TPA is not necessary to induce macrophagelike differentiation. Significant macrophagelike differentiation occurs in HL-60 cells after TPA exposure as short as 20 minutes.[61] Finally, unlike the granulocytic and monocytic HL-60 inducers, TPA induction is not confined to a small subset of fresh nonlymphocytic leukemia cells. Indeed, most acute myeloid leukemia cells as well as chronic myelogenous leukemia (CML) blast crisis cells exhibit this macrophagelike differentiation when exposed to TPA.[67-69]

There is a curious dose dependency of the TPA-induced phenotypic changes in HL-60 cells. At low doses of TPA (3×10^{-10} mol/L), HL-60 colony formation in soft agar in the absence of colony-stimulating factor (CSF) is enhanced ~ twofold (S. Collins, unpublished observations). Such colony formation is suppressed at TPA concentrations of $\geq 10^{-9}$ mol/L. This dose-dependent TPA stimulation is similar to that reported on the KG-1 myeloid leukemia cell line[70] as well as on fresh human myeloid leukemic cells[71] and on mouse bone marrow colony-forming cells.[72] At slightly higher concentrations (4×10^{-10} mol/L), TPA has induced granulocytic differentiation of HL-60 in liquid suspension culture.[73] Concentrations $>5 \times 10^{-10}$ mol/L induced the macrophagelike phenotype. Thus, at low concentrations of TPA, HL-60 proliferation is somewhat enhanced, at slightly higher concentrations granulocytic maturation occurs and, at still higher concentrations, the macrophagelike HL-60 phenotype is induced.

TPA exerts different phenotypic changes on HL-60 cells depending on the differentiative state of these cells. In HL-60 cells induced to granulocytes or monocytes TPA induces respiratory burst activity with the conversion of oxygen to superoxide anion.[52,74] This reaction forms the basis for the nitroblue tetrazolium (NBT) test, which is commonly used to quantitate HL-60 differentiation since undifferentiated HL-60 cells do not exhibit this particular response to TPA.[74,75] Similarly, TPA rapidly induces chemiluminescence in HL-60 cells induced to granulocytes but not in uninduced HL-60.[76]

In contrast to the granulocytic and monocytic HL-60 inducers considerable information is available related to the mechanism of action of TPA. HL-60 variant subclones have been isolated that are resistant to DMSO but that differentiate with TPA, suggesting that different pathways are involved in DMSO- and TPA-induced HL-60 differentiation.[77] TPA may exert many of its effects through protein kinase C, a calcium- and phospholipid-dependent enzyme that is widely distributed in numerous tissues and which plays a crucial role in transducing various extracellular signals across the cell membrane.[78,79] HL-60 cells harbor specific receptors for TPA,[80,81] and considerable evidence in HL-60 as well as in other cells shows that the TPA receptor is indeed protein kinase C.[82-84] Normally, protein kinase C is transiently activated by diacylglycerol, but TPA can substitute for endogenous diacylglycerol, resulting in prolonged activation of the enzyme.[85] This activation of protein kinase C in HL-60 cells could result in the phosphorylation of specific substrate proteins leading to the macrophagelike phenotypic changes induced by TPA. What are the possible substrates for activated protein kinase C in TPA-stimulated HL-60 cells? Following 1-hour treatment with TPA, increased phosphorylation of at least 14 proteins has been noted in HL-60.[86] Specifically, rapid phosphorylation of the HL-60 transferrin receptor occurs, associated with a decline in surface transferrin-receptor number.[87] In addition, phosphorylation of two cytosol proteins (17 and 27 kd in size) occurs in HL-60 cells within 15 minutes after exposure to TPA.[88] Activation of topoisomerase II by protein kinase C has been observed in vitro and this, together with the observation that TPA-induced HL-60 differentiation is partially blocked by inhibitors of topoisomerase II, suggests that this DNA-modulating enzyme could be an important target for activated protein kinase C in TPA-treated HL-60 cells.[89]

HL-60 variants stably resistant to TPA-induced differentiation have been derived by culturing the cells in gradually increasing concentrations of TPA.[90,91] These TPA-resistant HL-60 cells generally exhibit the same number of TPA receptors exhibited by wild-type HL-60.[90,91] Moreover, similar cellular proteins are rapidly phosphorylated after TPA exposure in both the TPA-sensitive and TPA-resistant HL-60 cells, but the quantitative level of this rapid phosphorylation is significantly less in the TPA-resistant *v* the TPA-sensitive cells.[92] These observations suggest that a block exists in the enzymatic activity of protein kinase C after its interaction with TPA in the TPA-resistant cells. This block may be related to the aberrant translocation of this enzyme from cytosol to membrane fractions that has been observed in the TPA-resistant HL-60 cells as compared with the TPA-sensitive HL-60 cells.[93]

The TPA-induced binding to, and activation of, protein kinase C appears to be important in the TPA-induced differentiation of HL-60 cells. Several observations suggest, however, that activation of protein kinase C cannot by itself account for this macrophagelike differentiation. For example, the synthetic diacylglycerol l-oleoyo-2-acetylglycerol (OAG) binds to and activates protein kinase C and induces the rapid phosphorylation of HL-60 cellular proteins.[86] This compound, however, does not induce HL-60 differentiation.[86] Similarly, the macrocyclic lactone bryostatin binds to and activates protein kinase C in HL-60 but actually inhibits TPA-induced HL-60 differentiation in a dose-dependent manner.[94] These observations suggest that a TPA effect other than, or in addition to, activation of protein kinase C must account for the macrophagelike differentiation of HL-60. The nature of this additional TPA action remains to be defined.

Eosinophils. Several observers have noted cells positive for the eosinophil specific Luxol-fast-blue stain in a small percentage (4% to 7%) of HL-60 colonies cloned in semisolid agar culture.[12,95] The proportion of colonies containing such cells increased with the addition of crude preparations of GM-CSF. Preparations containing EO-CSF, which stimulates the formation of normal eosinophil colonies, had no effect on the incidence of such colonies.[12] Similarly, purified recombinant GM-CSF induces a small but significant increase in the number of Biebrich scarlet cells in HL-60 liquid suspension cultures.[13] Moreover, variant HL-60 subclones exhibiting virtually 100% staining with Biebrich scarlet as well as other eosinophil characteristics have been isolated and stably maintained in continuous suspension culture.[96] Fischkoff et al noted that the proportion of cells exhibiting eosinophilic characteristics in the wild type HL-60 line could be markedly increased by culturing the cells in slightly alkaline media (pH 7.6 to 7.8).[97] These induced cells have been extensively characterized and demonstrate multiple eosinophilic granule proteins, including the eosinophil major basic protein (MBP), the Charcot-Leyden Crystal (CLC) protein, eosinophil peroxidase, acid phosphatase, and arylsulfatase.[97,98] Parental, uninduced HL-60 cells also exhibit the eosinophil MBP. The amount of this protein markedly diminishes when the culture is treated with granulocytic inducers such as DMSO.[97]

Some of the HL-60 eosinophilic characteristics are aberrant as compared with those of normal eosinophils. This is particularly true with regard to granule formation. For instance, the HL-60 eosinophilic granules are chloroacetate esterase and PAS positive, whereas normal eosinophilic granules are negative for both.[97] HL-60 eosinophils express significantly less CLC and arylsulfatase protein than do normal eosinophils.[98] Such abnormalities are similar to those observed in the dysplastic eosinophils present in the bone marrow of patients with acute myelomonocytic leukemia. This latter syndrome is associated with karyotypic abnormalities involving the long arm of chromosome 16,[99,100] and similar karyotypic abnormalities in this chromosome region have been noted in HL-60 metaphases.[97]

HL-60 ONCOGENE STRUCTURE AND EXPRESSION

HL-60 cells provide an invaluable model system for studying the relationship of oncogene structure and expression to normal and leukemic cell proliferation and differentiation. Several mutations involving specific cellular oncogenes have been noted in HL-60, reinforcing the concept that the development of malignancy is a multistage phenomenon, that cancer results not from a single mutation but from a series of mutations. In addition, studies on HL-60 have provided important insight into the relationship of specific oncogene expression to differentiation along a specific hematopoietic lineage.

HL-60 cells exhibit specific genomic mutations involving the cellular oncogenes c-*myc*, N-*ras* and p53. These are discussed separately.

*c-*myc *amplification.* The human c-*myc* gene is defined by virtue of its homology to v-*myc*, the transforming gene of the avian myelocytoma virus, which induces myeloid tumors in chicks. The protein product of this cellular oncogene is localized in the nucleus,[101] and enhanced expression of the c-*myc* gene is rapidly induced following mitogenic stimulation of resting lymphocytes or fibroblasts.[102] In addition, this oncogene acts in concert with other oncogenes, particularly those in the *ras* gene family, to malignantly transform primary rodent fibroblast cultures.[103] HL-60 cells exhibit a 15- to 30-fold genomic amplification of c-*myc* as compared with normal cells.[104,105] This observation was the first reported example of amplification of a cellular oncogene in any human malignancy. This c-*myc* amplification was also present in the fresh, uncultured leukemic cells of the patient from whom the HL-60 cell line was derived, indicating that this amplification did not arise during prolonged in vitro culture.[105] This c-*myc* amplification exhibited by HL-60 appears to be unusual among human hematopoietic malignancies. In a survey of >100 fresh human leukemias and lymphomas, Rothberg et al noted only one other hematopoietic malignancy (a Burkitt lymphoma) that exhibited genomic amplification of c-*myc*.[106]

In correlation with this genomic c-*myc* amplification, HL-60 cells exhibit high levels of steady state c-*myc* RNA. These levels significantly decrease as HL-60 cells are induced to differentiate terminally with granulocytic inducers.[107] In addition, monocytic and macrophagelike differentiation of HL-60 is associated with a marked decrease in c-*myc* RNA levels.[108,109] This decreased c-*myc* expression in differentiating HL-60 cells appears to mimic that which occurs in normal hematopoiesis since a similar decrease in c-*myc* RNA has been noted in normal bone marrow progenitor cells undergoing terminal differentiation in liquid suspension culture.[110]

Decreased c-*myc* transcription rather than enhanced c-*myc* mRNA degradation appears to account for the diminished steady-state c-*myc* RNA in differentiated HL-60.[111-113]

In retinoic acid-treated HL-60 this transcriptional downregulation occurs as a block in elongation of the *myc* RNA transcript between exons 1 and 2 rather than as decreased transcript initiation.[112] This novel mechanism of transcriptional downregulation might be particularly adapted to genes in which transcription is rapidly regulated and HL-60 cells provide the prototype model for studying this phenomenon.

N-ras. HL-60 genomic DNA is capable of malignantly transforming NIH3T3 cells in transfection assays, and the HL-60 gene that mediates this transformation has been identified as the N-*ras* gene,[114] a member of the *ras* family of oncogenes which also includes Kirsten and Harvey *ras*. Acquisition of NIH3T3-transforming ability frequently is associated with specific point mutations within the *ras* oncogenes, and a point mutation involving the second nucleotide of N-*ras* codon 61 has been documented in HL-60.[115] Other acute myeloid leukemia cells exhibit NIH3T3-transforming activity and N-*ras* point mutations.[116–118] In contrast to HL-60, these mutations usually involve N-*ras* codons 12 and 13.[116]

The normal cell function of the N-*ras* cellular oncogene is presently unknown. Why acute myeloid leukemia cells preferentially exhibit mutations in this particular *ras* gene rather than in other members of the *ras* gene family is also unclear. In contrast to c-*myc* gene expression, expression of the N-*ras* gene does not appear to change significantly as HL-60 cells are induced to differentiate. An approximately twofold decrease in N-*ras* RNA was noted in DMSO-differentiated HL-60,[119] whereas no change in N-*ras* expression was noted in TPA-treated HL-60 cells.[109]

p53. The p53 protein is frequently found at elevated levels in various types of tumor cells. This protein shares some characteristics with the c-*myc* product. Both are localized to the nucleus, and p53 can substitute for *myc* in the *myc–ras* cooperative transformation of primary rodent fibroblast cultures.[120,121] The p53 locus is on the short arm of chromosome 17,[122] and this gene is extensively deleted in HL-60 associated with absent p53 expression.[123] Moreover, the opposite p53 allele is absent, presumably secondary to the monosomy 17 that has been demonstrated in HL-60 metaphases.[4] The selective advantage if any of this p53 deletion is unknown. Because c-*myc* and p53 may share similar physiologic functions, it has been suggested that loss of p53 in progenitors of HL-60 may have led to the subsequent selection of cells harboring amplification of c-*myc*.[123]

Although the expression of cellular oncogenes was initially presumed to be associated with actively proliferating cells, some recent rather surprising observations indicate that expression of some cellular oncogenes is enhanced in certain terminally differentiated cells. Examples of this in HL-60 involved the *fos*, *fms*, *src* and *fgr* oncogenes.

c-fos. The c-*fos* gene is the homolog of v-*fos*, the transforming gene of the FBJ-murine sarcoma virus, which induces osteosarcomas in mice. The c-*fos* gene product is localized in the nucleus.[124] Mitogen stimulation of resting fibroblasts induces the rapid (within 5 to 10 minutes) onset of c-*fos* mRNA and protein product, suggesting that c-*fos* may play a role in rendering resting cells competent to divide.[125–127] It is somewhat paradoxical then that a similar rapid induction of c-*fos* is noted when HL-60 cells are treated with TPA,[128,129] since this compound also induces a rapid, pronounced inhibition of HL-60 proliferation.[61,62] Enhanced c-*fos* expression has also been noted during the monocytic differentiation of the mouse WEHI-3B cell line,[130] as well as in normal monocytes and macrophages.[131] Moreover, HL-60 cells induced to monocytes with vitamin D_3 also exhibit enhanced *fos* expression, although such *fos* expression occurs more gradually without the rapid initial c-*fos* expression noted in TPA-treated HL-60.[132] The role that c-*fos* plays in monocyte/macrophage differentiation is presently unclear. c-*fos* expression does not appear to be absolutely essential for monocyte differentiation since vitamin D_3 has been noted to induce monocytic differentiation of some HL-60 variant subclones without inducing enhanced c-*fos* expression.[133]

c-fms. The c-*fms* gene is the cellular homolog of the transforming gene of the McDonough strain of the feline sarcoma virus. The c-*fms* gene product appears to be identical to the cell membrane receptor for the monocyte/macrophage colony-stimulating factor (CSF-1).[134] HL-60 cells induced to differentiate to monocytes with vitamin D_3 or to macrophagelike cells with TPA express enhanced c-*fms* RNA levels as compared with uninduced HL-60 cells.[109] The kinetics of this enhanced expression is considerably slower than induced c-*fos* expression, with c-*fms* transcripts initially noted ~6 hours after inducer treatment and peaking at 24 to 48 hours following induction.[109] By means of immunologic reagents CSF-1 receptors have been detected on monocyte-induced HL-60 cells, but their numbers appear relatively small when compared with normal monocytes/macrophages (C. Rettermier, personal communication, July, 1986). No enhanced *fms* expression is noted in HL-60 cells treated with granulocytic inducers.[109]

c-src. The c-*src* gene is the cellular homolog of the transforming gene of the Rous sarcoma virus and codes for a cell membrane-associated protein ($pp60^{c\text{-}src}$) with tyrosine kinase activity. The levels of $pp60^{c\text{-}src}$ kinase activity are increased in HL-60 cells induced to macrophages, monocytes, or granulocytes with TPA, vitamin D_3, and DMSO, respectively.[135] Normal nonleukemic monocytes also exhibit relatively high levels of $pp60^{c\text{-}src}$ activity.[135] Terminally differentiated rat CNS neurons similarly exhibit relatively high levels of c-*src*.[136] The physiologic function of c-*src* in mature myeloid cells and neurons is presently unknown, but the high level of expression of this cellular oncogene in these terminally differentiated cell types indicates that its function is probably unrelated to active cell proliferation.

c-fgr. c-*fgr* is the cellular homolog of the transforming gene of the Gardner-Rasheed feline sarcoma virus. This gene is a member of the *src* family of oncogenes and codes for a cell membrane-associated protein with tyrosine kinase activity.[137] This gene is expressed in peripheral blood leukocytes with preferential expression in mature monocytes and granulocytes and little expression in B and T lymphocytes. Induction of HL-60 granulocytic differentiation is associated with a marked increase in the level of expression of c-*fgr* (K.

Robbins, personal communication, July, 1986). As with c-*src*, the physiologic function of c-*fgr* in the terminally differentiated HL-60 cells is presently unknown.

SUMMARY AND FUTURE STUDIES

How closely does the in vitro HL-60–induced differentiation mimic normal granulocyte/monocyte/macrophage differentiation? As emphasized above, differences exist between the induced HL-60 cells and normal granulocytes and monocytes. Whether this reflects an intrinsic defect in the karyotypically abnormal HL-60 leukemic cells or represents the failure of such in vitro manipulations to mimic the normal in vivo hematopoietic environment is unknown. A related question is whether the bipotential (granulocyte/monocyte) nature of the HL-60 promyelocytes has its counterpart in normal promyelocytes. Granulocytes and monocytes clearly share a common stem cell, as evidenced by the frequent observation of mixed granulocyte/monocyte colonies in CFU-c assays. This stem cell, however, is generally believed to be more immature than the heavily granulated, peroxidase-positive promyelocyte, whose progeny are usually assumed to be granulocytes. The behavior of HL-60 promyelocytes suggests that the normal promyelocyte may also be capable of differentiating to monocytes. In this regard, it is noteworthy that studies in which HL-60 cells are treated sequentially with granulocytic (retinoic acid) and monocytic (vitamin D_3) inducers indicate that early events in the induction of HL-60 promyelocytes to granulocytes or monocytes are shared by cells of both lineages.[138] Nevertheless, the caveat remains that the leukemic HL-60 promyelocyte is aberrant and does not truly reflect the differentiative potential of normal promyelocytes.

The synergistic effect of various naturally occurring compounds, including retinoic acid, tumor necrosis factor, DIFs, and the interferons on HL-60 cells may reflect some of the normal control mechanisms involved in hematopoiesis. Although the usual in vitro hematopoietic stem cell agar assays generally exploit the effect of single compounds such as CSF or erythropoietin on stem cell proliferation and differentiation, the situation in vivo is undoubtedly more complex. Normal hematopoietic stem cell proliferation and differentiation probably results from a complex array of synergistic inhibitory and stimulatory signals provided by lymphokines, monokines, vitamins, corticosteroids, interferons, and the various CSFs. HL-60 cells provide a convenient system for assessing the synergistic effect of such biologic response modifiers on granulocyte/monocyte/macrophage differentiation.

What is the relation between TPA-induced HL-60 and normal monocyte/macrophage differentiation? TPA is a plant derivative that appears to mimic the effect of naturally occurring compounds such as diacyglycerol in activating the protein kinase C family of enzymes. Conceivably, TPA is not so selective as such endogenous activators of these enzymes and might simultaneously activate a number of different cellular enzymes and targets. This "sledgehammer" effect might account for the unusual macrophagelike phenotype exhibited by TPA-treated HL-60 cells. TPA is a very active membrane stimulator and induces oxidase activity, chemiluminescence, and degranulation in both normal and induced HL-60 monocytes and macrophages. Such membrane effects may also be mediated through protein kinase C.[139,140] TPA may simultaneously induce both differentiation and membrane activation of HL-60 cells to account for the unusual phenotype of TPA-treated HL-60.

The recent isolation and analysis of protein kinase C cDNA clones indicates that at least three distinct types of this enzyme are present in mammalian species and that expression of these different enzymes varies from tissue to tissue.[141-143] It will be of interest to determine the distribution of these different protein kinase C enzymes in uninduced *v* induced HL-60 cells. Conceivably, these various protein kinases may act on different substrates, may have different affinities for TPA, and may be differentially distributed in uninduced *v* induced HL-60. This might account for the different phenotypic changes induced in HL-60 by different concentrations of TPA and might also explain the different effects induced by TPA depending on the state of differentiation of the HL-60 cells.

The pronounced antiproliferative effect of TPA on HL-60 is of particular interest and could have therapeutic relevance, since a similar TPA-induced effect is also noted in most fresh acute myelogenous leukemia cells. The adverse effects (including tumor promotion) exerted by TPA on other cell types, however, rules out its current use as a therapeutic agent.[144] Future studies attempting to define the specific HL-60 TPA targets that trigger this growth inhibition should provide a greater understanding of the antiproliferative effects of TPA and may lead to more selective therapeutic agents for myeloid leukemia.

The HL-60 cell line, because of its distinct monocyte/granulocyte characteristics, will continue to provide a ready source of specific human mRNA for use in cloning important granulocyte and monocyte enzymes and monokines. Examples of such monocyte/granulocyte specific clones derived from HL-60 to date include the gene for tumor necrosis factor (cachectin) cloned from a TPA-treated HL-60 cDNA library[145] and the candidate gene that is defective in chronic granulomatous disease, cloned from a granulocyte-induced HL-60 cDNA library.[146]

HL-60 cells exhibit structural abnormalities of at least three different cellular oncogenes (c-*myc*, N-*ras*, and p53) and one hematopoietic growth factor (GM-CSF). How such mutations are related to the continuous proliferation and block in differentiation exhibited by HL-60 is an area of active investigation. Of particular experimental interest is the relationship between the HL-60 c-*myc* amplification and this block in differentiation. Does the induction of decreased levels of c-*myc* RNA per se lead to differentiation of HL-60 cells or are other direct or indirect effects of differentiation inducers on other HL-60 genes required: An "antisense" 15-base pair (bp) oligonucleotide complementary to the translation initiation region of c-*myc* was recently noted to induce differentiation of HL-60 cells (J. Holt, personal communication, December, 1986). The control "sense" oligomer was ineffective in inducing such differentiation. These observations are preliminary but suggest that the c-*myc* product is critical to maintaining HL-60 cells in a relatively undifferentiated proliferative state and that inhibition of this product alone may result in the terminal differentiation of HL60 cells. The normal physiologic function of most of the cellular oncogenes remains to be defined. Particularly perplexing are those oncogenes such as *fgr, fos,* and *src* whose expression is enhanced in certain terminally differentiated rather than actively proliferating cells. How closely HL-60 leukemic cell differentiation mimics the differentiation of normal myeloid progenitor cells is presently unclear, and significant differences between the mechanisms of HL-60 and normal myeloid progenitor cell differentiation may exist. Nevertheless, the specific changes in *myc, fos, fgr,* and *src* cellular oncogene expression that characterize HL-60 cell differentiation also appear to occur during normal myeloid cell differentiation. Thus, HL-60 cells should continue to provide an excellent model system for studying both the regulation of expression of these cellular oncogenes and the physiologic role these particular genes play in the terminal differentiation of normal monocytes, macrophages, and granulocytes.

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REVIEW ARTICLE

Hemoglobin S Gelation and Sickle Cell Disease

By William A. Eaton and James Hofrichter

THE FUNDAMENTAL cause of sickle cell disease is the decreased deformability of the sickled red cell produced by gelation of hemoglobin S. Partial inhibition of gelation should therefore reduce clinical severity, while complete inhibition should result in a "cure." These basic ideas have stimulated an enormous effort to understand the gelation process in detail and to relate the results of these studies to the pathophysiology of sickle cell disease. Discoveries concerning gelation have also led to new lines of research on a specific therapy. The early finding that fetal hemoglobin inhibits gelation.[1,2] has ultimately led to the development of methods to increase the production of F cells in the bone marrow of sickle cell patients,[3-5] while the discovery of the enormous sensitivity of the rate of gelation to hemoglobin concentration[6] has stimulated studies on the reduction of intracellular hemoglobin concentration as a means of therapy.[6-12] Studies on the structure of the hemoglobin S polymer,[13-18] moreover, have guided the development of agents designed to inhibit gelation by interfering with the formation of intermolecular contacts in the polymer.[19-21]

The purpose of this article is to review recent developments in the relation between hemoglobin S gelation and sickle cell disease. We first present our current understanding of the major features of the gelation process. Since gelation is a physical rather than a chemical process, its description necessarily requires more physical detail than that of most biological processes. From these studies we are able to develop a more rigorous and comprehensive description of the relation between gelation and the pathophysiology than has been possible up to now. By combining the gelation studies with work on the rheology of sickle cells and blood flow in the microvasculature, a clearer picture emerges of the outstanding issues in understanding the mechanism of vaso-occlusion in patients and the resulting cardiovascular response. Finally we discuss the variation in clinical severity and analyze the problem of inhibiting gelation in patients. Throughout this discussion we shall see that the kinetics of gelation is a dominant factor in understanding gelation both in vitro and in vivo, and it will become clear that discussions of the pathophysiology that do not include a kinetic analysis[22,23] are inadequate.

A broader treatment of sickle cell disease, including genetic and clinical aspects, has recently appeared in two excellent books.[24,25] Also the structure, physical chemistry, and rheology of hemoglobin S gelation in solution and in red cells is discussed much more extensively in an article that is being published elsewhere.[26]

GELATION AT EQUILIBRIUM

To understand gelation we first must describe a gel at equilibrium. As shown in Fig 1, a gel can be separated into two phases, a solution phase that contains free hemoglobin molecules and a polymer phase. The structure of the individual polymers has now been determined in considerable detail. It is a fiber made up of 14 intertwined helical strands of hemoglobin S molecules.[14,15,27] The fiber can alternatively be described in terms of seven intertwined double strands of molecules in which the double strands have a structure that, except for a slight helical twist, is nearly identical to the double strands that form the fundamental unit of the deoxyhemoglobin S single crystal.[16,28,29] In each molecule one of the two $\beta 6$ valines of the $\alpha_2\beta_2$ tetramer is involved in an intermolecular contact with its neighbor in the double strand. The structure of the deoxyhemoglobin S crystal is known to atomic resolution, so that there is a very detailed picture of the intermolecular contacts within the double strand that must be very similar to what occurs in the polymer.[16-18]

A gel at equilibrium behaves very much like a suspension of microscopic protein crystals suspended in a saturated protein solution.[30-32] The concentration of hemoglobin in the solution phase, which is called the solubility, is an accurate measure of the stability of the polymer phase. The solubility is determined experimentally by measuring the hemoglobin concentration in the supernatant obtained after high-speed sedimentation of the polymers.[31-36] Because the concentration of hemoglobin in the polymer phase appears to be relatively constant,[37] the fraction of the total hemoglobin that is polymerized can be calculated from the solubility using a simple mass conservation relation. There have now been systematic investigations of the solubility under a wide variety of solution conditions. These include the dependence on temperature,[31,32,35] pH,[38,39] salts,[40] 2,3-DPG,[39,41,42] carbon monoxide,[36,43,44] oxygen,[45,46] and non-S hemoglobins.[30,37,38,47-63]

The role of non-S hemoglobins in the gelation of hemoglobin mixtures has been the focus of a large number of studies, since early investigations showed that the presence of hemoglobins A, C, and F reduces sickling and is accompanied by decreased clinical severity.[25] For mixtures of hemoglobins S and F (and $S + A_2$), a detailed analysis of solubility data, including the large contribution of nonideality arising from excluded volume effects,[32,37,54,55] indicates that over the physiologic range of compositions there is little or no copolymerization of either the homotetramers, $\alpha_2\gamma_2$ (and $\alpha_2\delta_2$), or the hybrid tetramers, $\alpha_2\beta^S\gamma$ (and $\alpha_2\beta^S\delta$).[26,37,64] The low probabilities for copolymerization of these molecules can be rationalized as resulting from destabilizing effects on the intermolecular contacts of the double strand that accompany specific

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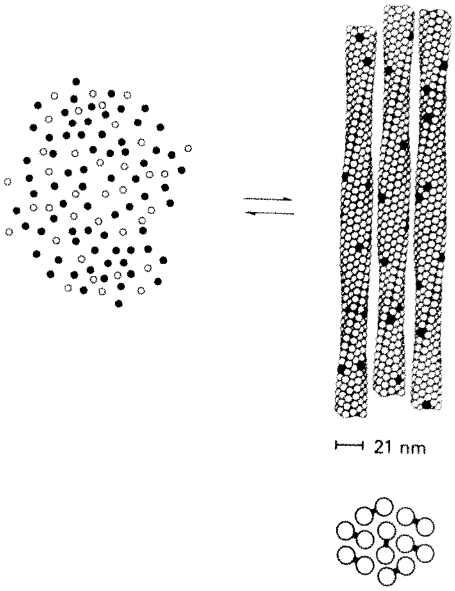


Fig 1. Schematic picture of a gel of hemoglobin S at partial saturation with oxygen. The 64 kD molecule (ie, the tetramer) is represented as a circle. A gel of hemoglobin S contains large polymers, often called fibers, and a concentrated solution of free hemoglobin molecules. The filled circles represent hemoglobin S molecules with one or more oxygen molecules bound. There are relatively fewer filled circles in the polymer because it has a lower oxygen affinity than the solution. The structure was determined by Edelstein and coworkers using electron microscopy and image reconstruction techniques.[14,15] The cross-section shows that the fiber consists of 14 strands and that it can be constructed from seven double strands that are very similar to those found in the deoxyhemoglobin S single crystal.[18,27,29]

amino acid replacements on the molecular surface.[37,65] For S + A and S + C mixtures the analysis indicates that there is little or no copolymerization of the $\alpha_2\beta_2^A$ and $\alpha_2\beta_2^C$ molecules but that the hybrid molecules $\alpha_2\beta^S\beta^A$ and $\alpha_2\beta^S\beta^C$ copolymerize with a probability that is approximately half that for $\alpha_2\beta_2^S$.[26,37,62] The factor of 2 is consistent with the structural result that a valine residue is required at only one of the two $\beta 6$ sites on each molecule for it to be incorporated into the polymer.

Binding of oxygen to hemoglobin S has a dramatic effect on gelation. Experiments performed over 35 years ago demonstrated that fully deoxygenated hemoglobin S gels, while fully oxygenated hemoglobin S does not.[66] To begin to consider the pathophysiology of sickle cell disease, however, it is clear that one needs quantitative data on solutions that are partially saturated with oxygen, particularly over the range of fractional saturations encountered in vivo. Such data have been obtained only relatively recently. The schematic picture of gelation in Fig 1 points out several important questions that must be answered regarding gelation in the presence of oxygen. These include (1) what is the fraction of hemoglobin molecules that are polymerized? and (2) what are the fractional saturations with oxygen of the molecules in the solution and polymer phases? The fraction polymerized can be determined from measurements of the solubility as a function of solution phase saturation.[46] Also, because there is no aggregation in the solution phase of the gel[67,68] and the $\beta 6$ mutation has no effect on the intrinsic affinity of the hemoglobin molecule, the binding curve for the solution phase hemoglobin S molecules is normal.[69,70] The major problem has been to obtain the polymer binding curve, which was accomplished using an optical technique called linear dichroism.[14,46,71-73]

The principal experimental results are shown in Fig 2. The solubility increases slowly at low oxygen saturation, then increases sharply at high saturations (Fig 2 b). The most interesting finding from the binding studies is that the polymer binds oxygen noncooperatively, as evidenced by a slope of unity in a Hill plot.[46] The two-state allosteric model,[74] which has provided an excellent framework for interpreting a wide variety of experiments on hemoglobin, provides a simple molecular interpretation of these results.[46] According to this model a hemoglobin molecule free in solution exists in one of two affinity states at all stages of oxygenation. The low-affinity state, called T, has the quaternary structure of completely deoxygenated hemoglobin, while the high-affinity state, called R, has the quaternary structure of the fully oxygenated molecule. Binding to either quaternary structure is noncooperative. Cooperativeness arises from the continuous conversion of low-affinity T-state molecules to high-affinity R-state molecules as the saturation increases (Fig 2 a). The simplest extension of this model to the gelation of hemoglobin S is to postulate that all T-state molecules polymerize with equal probability independent of the number of oxygen molecules bound and that there is no polymerization of R-state molecules.[46] The model is based on the idea that R-state molecules do not polymerize because their structure is sufficiently different from T-state molecules that they cannot fit into the polymer lattice. Analysis of the structure of the double strand of the deoxyhemoglobin S crystal does indeed show that it is impossible to replace the T-state molecule with an R-state molecule.[18] Since the polymer contains only T-state molecules, the model predicts that it will bind oxygen noncooperatively, exactly as observed. The model is not quantitatively perfect, however, because the affinity of the polymer is slightly lower than that of solution T-state molecules (Fig 2 a). Evidence for this small difference is also found in the solubility data (Fig 2 b), indicating that T-state molecules with oxygen bound are partially discriminated against by polymers. The small conformational changes that are known to take place within the T quaternary structure upon oxygen binding could explain this result.[45] The picture that emerges, then, is that the simplest extension of the two-state allosteric model provides an excellent description of the effect of oxygen on gelation.

The results of these experiments can be used to explain data on polymerization in sickle red cells. The first step is to consider further the oxygen binding curve of a gel. As mentioned above, at a given oxygen pressure the saturation of the gel is a weighted average of the saturations of the solution and polymer phases. Figure 2 c shows gel binding curves under near physiologic conditions that have been constructed from solution and polymer phase binding curves (Fig 2 a) and from the solubility curve (Fig 2 b). As the

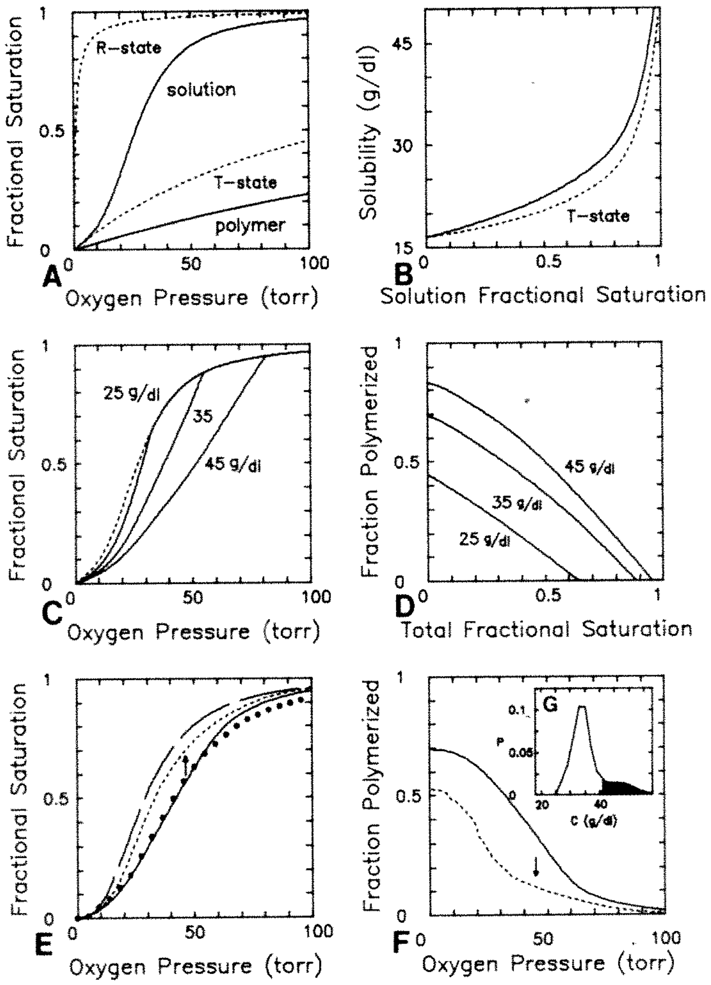


Fig 2. Effect of oxygen on gelation in solution and in sickle cells. (A) Solution, polymer, and theoretical R- and T-state binding curves. The solution binding curve is the binding curve for normal blood. The polymer binding curve is calculated from the data in phosphate buffer of Sunshine et al.[46] The R- and T-state binding curves are theoretical and were obtained by fitting to the solution binding curve with the two-state allosteric saturation function.[74] (B) Solubility as a function of solution-phase saturation and oxygen pressure from the data of Sunshine et al.[46] The dotted curve shows the theoretical solubility for the hypothetical case in which all T-state molecules polymerize with equal probability, independent of the number of oxygen molecules bound. (C) Gel-binding curves at different total hemoglobin concentrations calculated from the results in (A) and (B).[28,46] The dotted curve is the solution binding curve of A. (D) Fraction polymerized as a function of total fractional saturation calculated from the results in (A) and (B).[28,46] (E) In vivo oxygen binding curve calculated for a population of cells having the distribution of intracellular hemoglobin S concentrations in (G). The binding curve calculated in the absence of polymer (long-dashed curve) and the binding curve calculated when polymer is present at equilibrium (solid curve) are shown for reference. The data points are for SS blood from Winslow.[77] The oxygen unloading curve under in vivo conditions (dotted curve) was calculated by requiring that only the densest 18% of the cell population contain polymer at the average venous Po_2 found in SS patients (46 torr)[78] denoted by the arrow. These conditions were simulated by requiring that each cell be sufficiently supersaturated for polymerization to occur within about 200 ms at each Po_2. (F) Fraction of total hemoglobin S that is polymerized. The fraction is calculated under equilibrium conditions (curve) and under in vivo conditions (dotted curve). (G) The average distribution of intracellular concentrations from a study of 43 patients by Fabry et al[75] used in the calculations in panels (E) and (F). The probability density, P, in dL/g is plotted *v* the intracellular hemoglobin concentration, C, in g/dL. The blackened area shows the subpopulation of cells that contain polymer at Po_2 of 46 torr. The equations and parameters used in the calculation of all of the above panels are given by Eaton and Hofrichter,[28] which are derived from the work of Sunshine et al.[46]

oxygen pressure increases, not only do the saturations of both phases increase but the solubility also increases, decreasing the contribution of the low affinity polymer phase to the total binding curve. As a result the gel binding curves appear to have lower-than-normal affinity with higher-than-normal cooperativeness.[44] At sufficiently high oxygen pressures the solubility exceeds the total hemoglobin concentration, and the binding curve superimposes on the normal solution binding curve (Fig 2 c). As the total hemoglobin concentration increases, the affinity of the gel decreases owing to the increased fraction of the low affinity polymer (Fig 2d), and the oxygen pressure at which the polymer disappears also increases.

The oxygen-binding curve of a single red cell should be identical to the binding curve of a gel having the same composition (total hemoglobin S concentration, fraction fetal hemoglobin, pH, 2,3-DPG concentration, etc). Oxygen-binding curves of sickle blood are an average of the gel-binding curves for the individual cells. As in gels, the binding curve for sickle blood is "right shifted" compared to normal blood. Although 2,3-DPG levels are elevated in sickle cell blood, the formation of the low-affinity polymer is the major cause of the right shift in the blood-binding curve.[77,79]

The major difference between the binding curves for cell suspensions (Fig 2 e) and for gels (Fig 2 c) results from the wide distribution of hemoglobin S concentrations, which varies from about 20 g/dL in F cells to almost 50 g/dL.[79-81] This distribution produces a wide range of median affinities within the red cells from a given patient and hence smears the characteristic features of the gel-binding curve (compare Figs 2 c and 2 e). To calculate blood-binding curves it is necessary to utilize the results of recent investigations that have characterized the distribution of total hemoglobin concentrations from density measurements.[62,75,76,82-88] The density distributions for SS cells are broader and more variable than those for normal individuals. Since the distribution of intracellular hemoglobin S and hemoglobin F concentrations was not determined for the cells employed in the oxygen-binding measurements, it is only possible to make qualitative comparisons between the observed binding curves and those calculated from solution data. Figure 2 e compares a whole blood oxygen-binding curve with the binding curve calculated from the solution data using the average concentration distribution from a study of 43 patients.[26,75] The p50s for the curves calculated from concentration distributions for individual patients vary from 37 torr to 46 torr, compared to the 33 torr to 45 torr observed in a study of 14 patients.[77,89] This comparison shows that the patient-to-patient variability in oxygen binding curves can be readily accounted for by the variability in intracellular concentration distributions. A more direct comparison on a relatively homogeneous cell population obtained by density fractionation, in which the 2,3-DPG and hemoglobin F levels were also measured, gives very good agreement between the cell and solution data.[26,79]

Gelation in isolated solutions and cells has also been compared using nuclear magnetic resonance techniques to measure the average fraction of polymerized hemoglobin as a function of the total saturation of the cells.[76,90-92] The nuclear magnetic resonance measurements take advantage of the fact that the polymerized molecules do not rotate freely, making it possible to selectively measure the spectra of the polymerized and unpolymerized molecules.[93] Measurements on a cell population of known concentration distribution are in good agreement with the curve calculated from the solution data.[76] Agreement is also obtained in a comparison of density fractionated cells, although the experimental uncertainties are much larger.[76]

At this point we should emphasize that the oxygen binding curves and polymer fraction curves that we have discussed are equilibrium or near-equilibrium curves and, as we shall see later, are very different from the in vivo situation in which most cells are very far from equilibrium because of the large kinetic effect of the delay time (Fig 2 e and 2 f).

KINETICS AND MECHANISM OF GEL FORMATION

The most unusual and interesting aspect of the gelation process is the kinetics and mechanism of gel formation. The simplest kinetic experiment takes advantage of the characteristic property that a hemoglobin S solution gels upon heating. A completely deoxygenated solution, having a concentration significantly less than the solubility at 0°C (< 30 g/dL), is heated to some temperature where the concentration exceeds the solubility. Polymer formation can be detected by a variety of techniques, including linear birefrin-

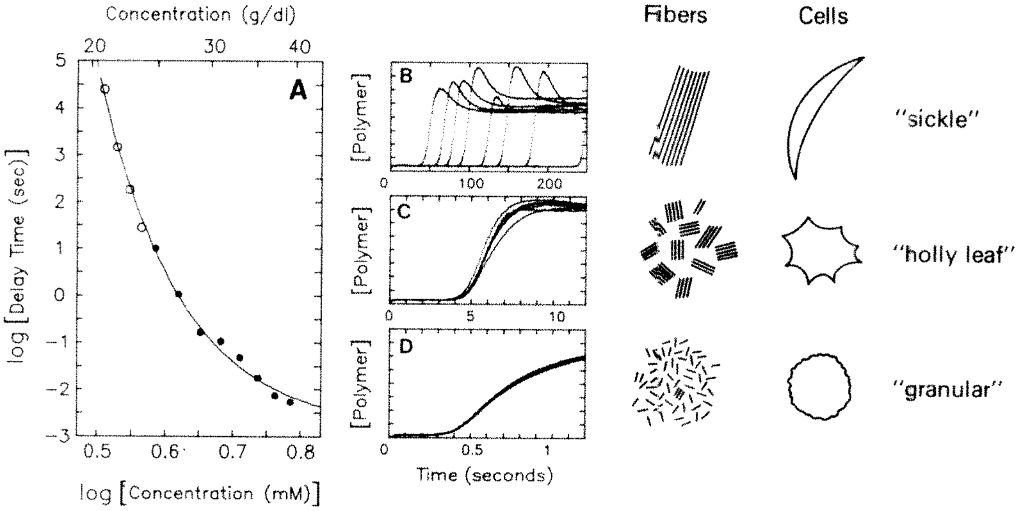
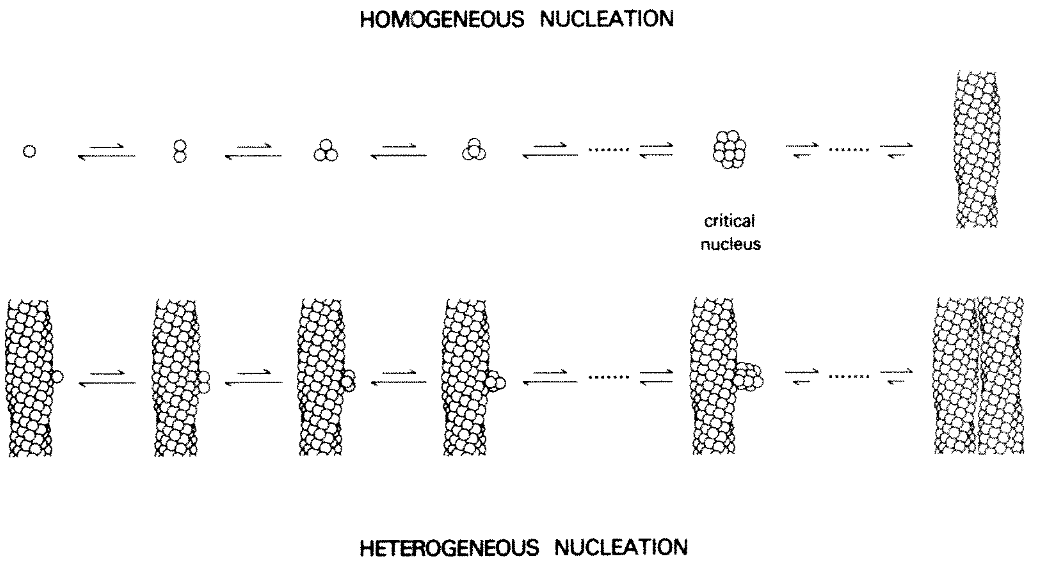


Fig 3. Kinetics of gelation and morphology of cells. (A) Concentration dependence of the delay time from laser photolysis (filled circles) and temperature-jump measurements (open circles; from Ferrone et al[132]). (B, C, D) Reproducibility of kinetic progress curves for samples having different delay times (from Hofrichter[133]). The schematic at the right shows that in a slowly polymerizing cell the "sickle" morphology is postulated to result from the formation of a single domain of well-aligned fibers; that in a more rapidly polymerizing cell the "holly leaf"morphology results from the formation of a number of smaller domains of shorter, aligned fibers; and that in the fastest polymerizing cells the "granular" morphology results from the formation of a large number of very small domains or randomly oriented short fibers.

gence,[6,43,45,94] turbidity,[36,37,43,44,57,95-112] light scattering (G.W. Christoph and R.W. Briehl, unpublished results),[113-115] viscosity,[59,112,116-122] water proton magnetic resonance linewidths[123] and transverse relaxation times,[124-128] and electron paramagnetic resonance.[129] All of the techniques show the same type of time course. There is an apparent delay period during which there is no evidence for any aggregation, followed by the explosive appearance of polymer.[6,94,95,116,130] Upon lowering the temperature of a preformed gel, depolymerization proceeds much more rapidly and without a delay.[6,94,95] The most striking finding from these studies is that the delay time is enormously sensitive to solution conditions, in particular to the hemoglobin S concentration. The inverse of the delay time is found to be proportional to the 30th to 50th power of the initial hemoglobin concentration.[6,36,37] This is the highest known concentration dependence for a process taking place in solution. The delay time is also found to be directly proportional to the 30th to 45th power of the solubility, independent of the manner in which the solubility is altered.[36,97] For example, in temperature-jump experiments, the delay time for a solution that is 10% saturated with carbon monoxide is increased by a factor of about 10 relative to deoxyhemoglobin S at the same total hemoglobin concentration, and at a saturation of 40% the delay time is increased by a factor of about 10^4.[36,44]

The temperature-jump technique is limited to measuring delay times longer than about 100 seconds. To extend the kinetic measurements to physiologic times and hemoglobin concentrations required the development of a laser photolysis technique that could be used to prepare a completely deoxygenated hemoglobin S solution in less than a few milliseconds.[131-133] In this technique the carbon monoxide complex of hemoglobin S, which is soluble up to at least 48 g/dL,[134] can be converted to deoxyhemoglobin S by photodissociation under continuous laser illumination. The laser also serves as a source for monitoring gel formation from the change in light scattering. When the laser is turned off, the carbon monoxide recombines, the polymer disassembles to form a solution of monomers (ie, 64 kD hemoglobin S tetramers), and the experiment can be repeated indefinitely. Because the volumes of observation are as small as 10^{-11} cc, the laser photolysis technique can also be used for investigating gelation in single red cells.[135-137]

A combination of the temperature jump and laser photolysis techniques has been used to examine the kinetics of gelation over a wide range of concentrations, temperatures, and times.[132] Figure 3 shows that as the concentration decreases, the delay time increases from about 10 milliseconds at 40 g/dL to about 100,000 seconds at 20 g/dL. An important clue to the mechanism by which gelation occurs comes from a very unusual result, discovered in the course of the laser photolysis experiments, which is described in Fig 3. Highly reproducible delay times are observed for solutions with delay times of a few hundred milliseconds or less. When the delay times become longer than a few seconds, however, the delay times become very *ir*reproducible, despite the fact that the progress curves have very similar shapes once polymerization begins (Fig 3 b to d).[131-133] An important companion observation is that only a single birefringent domain of polymers forms when there are large fluctuations


HOMOGENEOUS NUCLEATION

HETEROGENEOUS NUCLEATION

Fig 4. The double nucleation mechanism (Ferrone et al[138]). The two pathways for nucleation of polymers are shown. In the homogeneous pathway nuclei form in the solution, while in the heterogeneous pathway nuclei form on the surface of existing polymers. As more polymers form the increased surface area results in a continuously increasing rate of heterogeneous nucleation. This autocatalytic formation of polymers via the heterogeneous nucleation pathway is responsible for the appearance of a delay period prior to the observation of polymer. For both nucleation pathways there are competing thermodynamic forces. Initially aggregation is unfavorable because entropic forces tend to keep molecules apart. As the nuclei become larger, however, there is an increased number of bonds per monomer, 1/2 for a dimer, 3/3 for a trimer, 6/4 for a tetramer, up to 4.1 in the infinite polymer. As the aggregates grow this increase in the stability from more bonds per monomer finally overcomes the unfavorable entropic forces. The aggregate for which addition of monomer finally becomes favorable is called the critical nucleus.

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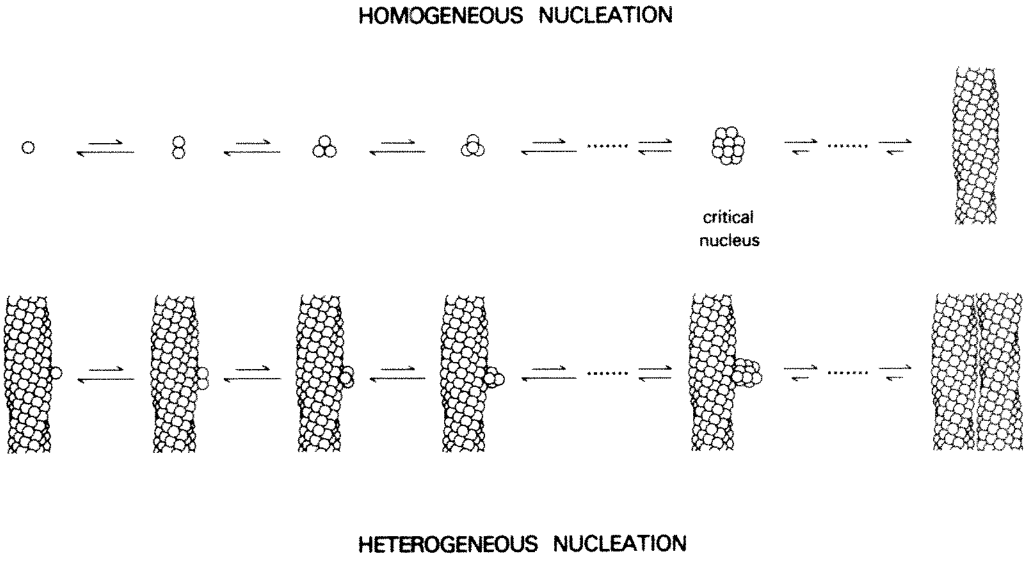
HOMOGENEOUS NUCLEATION

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inside sickle cells proceeds at the same rates and by the same mechanism as in purified solutions. Several results indicate that the answer is *yes*. First, studies on the addition of red cell membrane components to deoxyhemoglobin S solutions show little or no effect on the delay time.[110,111] Second, the laser photolysis technique has permitted the measurement of the kinetics of gelation in single red cells, yielding results that are in qualitative agreement with those predicted from the solution studies.[135] The shapes of the kinetic progress curves are very similar to those observed in solution,[135-137] and for slowly polymerizing cells there are the expected stochastic fluctuations in the delay times.[135] Figure 6 shows that the distribution of observed delay times, which range from a few milliseconds to over 100 seconds, is almost exactly what is predicted from the solution studies and the known concentration heterogeneity. A more detailed comparison of solution and cell data can be made by calculating the intracellular hemoglobin S concentration distribution from the delay time distribution using the solution delay times. The calculated distribution in Figure 6 is qualitatively the same as the measured distributions (Figure 2g), showing that there are no major differences in the rates of gelation in solution and in cells.

SICKLING AS AN INDICATOR OF INTRACELLULAR GELATION

While it has long been accepted that the deformation of SS red cells upon complete deoxygenation is caused by intracellular polymerization,[66] the detailed relation between gelation and cell deformation has remained somewhat ambiguous. In this section we address two questions. The first is whether or not there is a well-defined relationship between cell shape and intracellular polymerization. The second is whether the wide variety of observed cell shapes can be rationalized in terms of what we now know about the kinetics and thermodynamics of gelation. The answers to these questions are important, since a direct link between cell morphology and polymerization would permit a variety of experiments to be performed by using morphological criteria in place of more complex and difficult physical measure-

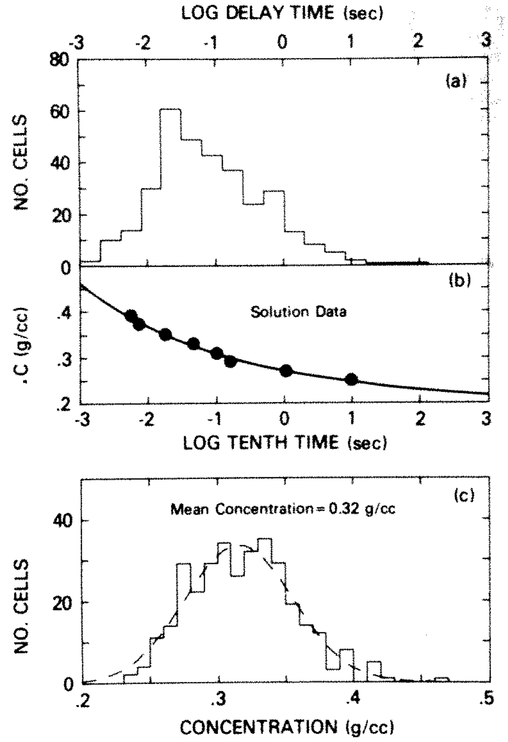

Fig 6. **Distribution of delay times for deoxyhemoglobin gelation in individual cells (from Coletta et al[135]). (A) Distribution of delay times from three homozygous SS patients. (B) Relation between concentration and logarithm of tenth time from solution data (Fig 3). (C) Intracellular hemoglobin S concentration distribution determined by calculating the concentration corresponding to each delay time in (A) using the result in (B). The intracellular hemoglobin S concentration is somewhat underestimated because the delay times in potassium phosphate buffer are shorter than in physiologic buffer (P.L. San Biagio, J. Hofrichter, and W.A. Eaton, unpublished results).**

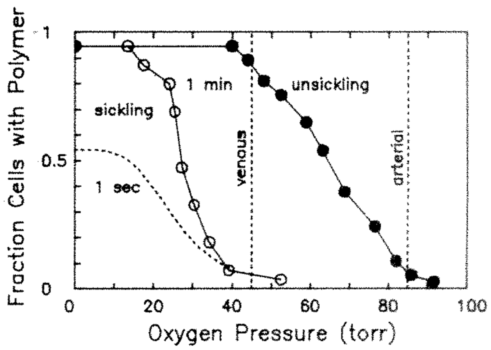

Fig 7. **Fraction of cells containing polymer as a function of calculated oxygen pressure determined by a double laser beam photolysis technique (from Mozzarelli et al[136,137]). In this technique one laser beam illuminating a single red cell is used to prepare hemoglobin S at a steady-state partial saturation with carbon monoxide by continuous photodissociation, while a second more intense laser beam can be switched on at any time to completely photodissociate the remaining carbon monoxide and to measure the kinetics of gelation from the time course of the scattered laser light. If no polymerized hemoglobin is present in the cell at partial saturation, the kinetics of gelation at zero saturation (produced by the second laser beam) are characterized by a delay period, while the presence of polymerized hemoglobin is indicated by the loss of the delay period. Experiments on hemoglobin S solutions show that this technique accurately simulates the gelling behavior of hemoglobin S at partial saturation with oxygen and that the presence of as little as 0.05% polymerized hemoglobin results in a marked shortening of the delay period. The oxygen pressures were calculated from the measured saturations with carbon monoxide using the least squares fit of the two-state allosteric saturation function to the binding curve of normal blood. The filled circles are the equilibrium data obtained in reoxygenation (ie, resaturation with carbon monoxide) experiments, the open circles are data obtained from experiments where deoxygenation (desaturation) is carried out over a period of one minute, and the dashed curve is a theoretical estimate for deoxygenation carried out in one second.[141] The vertical dashed lines indicate the average oxygen pressure found in the arteries and veins of patients with homozygous SS disease.[78]**

ments such as light scattering[135-137] or micropipette measurements.[140]

The relationship between cell deformation and intracellular polymerization has recently been studied with a variation on the laser photolysis technique. Gelation in partially saturated single cells was investigated by using the kinetics of gelation after complete photodissociation as a probe for the presence of polymer (Fig 7).[136,137] The delay time provides a very sensitive probe for polymer because even vanishingly small amounts of polymerized hemoglobin ($< 0.05\%$) drastically reduce or eliminate the delay period.[136,137] These experiments demonstrate clearly that sickling accurately reflects the onset of gelation and that unsickling indicates the complete disappearance of polymer.[136,137] As a result, curves that describe the fraction of cells containing polymer as a function of saturation or oxygen pressure may be designated sickling and unsickling curves. Figure 7 shows the fraction of sickled cells, ie, the cells that contain polymerized hemoglobin S, as a function of the oxygen pressure, calculated from the measured saturation with carbon monoxide. There is a very large hysteresis between the sickling and unsickling curves. The oxygen pressure at which polymers are first observed in deoxygenation experiments in an initially polymer-free cell is always much lower than the pressure at which polymers disappear in reoxygenation experiments.The hysteresis occurs because there is a delay period before polymer can be detected upon deoxygenation, but in reoxygenation experiments depolymerization occurs without any delay period. Thus the unsickling curve is very close to an equilibrium curve, while the sickling curve depends on the rate of deoxygenation. The sickling curve in Fig 7 was measured by lowering the saturation over a period of one minute. In the microcirculation, deoxygenation occurs in about one second; sickling curves have not yet been measured on this time scale. However, the theoretical one-second sickling curve shown in Fig 7 is seen to be extremely left shifted,[141] in qualitative accord with the results of kinetic studies that show that about 50% of cells sickle after about one second at zero oxygen pressure.[142,143] Experiments in which cellular deformation occurs much more rapidly have also been performed by deoxygenating cells in a mixer.[144] These data are consistent with the results on intracellular gelation using the laser photolysis technique,[26,135] suggesting that cell deformation is a reliable indicator of intracellular gelation on the second and subsecond time scale as well.

Essentially identical conclusions have been reached from experiments in which rheologic techniques are used to examine individual sickle cells. The most direct data have come from measurements as a function of oxygen pressure using micropipette techniques.[140] Both the static and dynamic rigidities of the cells can be measured. The static rigidity is characterized by the change in the length of the "tongue" aspirated into the pipette with a change in negative pressure, while the dynamic rigidity is characterized by the half-time required to achieve the final tongue length after initiating the pressure change.[145] For oxygenated cells there are only small increases in these quantities, with the largest increases for the irreversibly sickled cells and the densest cells.[146,147] As the oxygen pressure is decreased, cells are observed to undergo a variety of morphological changes, and cells having a given set of morphological characteristics can be examined.[140] Cells that are "spiculated" or have a "granular" surface show markedly altered rheology. In contrast, discocytes that maintain a "smooth surface" show the same static and dynamic rigidities at all oxygen pressures as normal cells, in agreement with the conclusion from the kinetic studies. In cells showing morphologic evidence of gelation, both the static rigidity and the half-time for tongue growth increase with decreasing oxygen pressure. At the lowest oxygen pressures the static rigidity increases by up to a factor of 100, and the half-time for tongue growth increases by a factor of 150 to 1000 relative to normal cells and oxygenated sickle discocytes.[140,148] Since both parameters are much greater for all cells containing polymerized hemoglobin than for polymer-free cells, it appears that the presence or absence of intracellular polymer is much more important in determining cellular rigidity than the extent of intracellular polymerization.[149]

A closely related problem is to understand the enormous variety of cell shapes that are observed in a population of sickled cells. It has been known for over 45 years that slow deoxygenation results in elongated, birefringent cells, while rapid deoxygenation produces a much less distorted cell, originally called a granular form.[150] An unexpected bonus provided by the double nucleation mechanism is that it suggests an explanation for these observations.[138] When cells are rapidly deoxygenated the solubility is suddenly decreased to a low value. The resulting high supersaturation (the ratio of the total concentration to the solubility) causes a high rate of homogeneous nucleation, and the resulting gel contains a very large number of small polymer domains or randomly oriented polymers that could give the cell a granular appearance (Fig 3). In contrast, when deoxygenation is slow, the rate of homogeneous nucleation is reduced to the point that only one homogeneous nucleation event takes place in the cell, and a single polymer domain forms. If it were not for the limited amount of hemoglobin in the cell, this domain would grow to a much larger size than the cell. The cell membrane presumably restricts domain growth to one general direction, resulting in an elongated cell with approximately parallel polymers, the classic "sickle" form (Fig 3). At intermediate rates of deoxygenation, cells may contain a countable number of domains, where, for example, each domain could produce one of the projections of a so-called "holly-leaf" shape (Fig 3).

The appearance of a wide range of morphological forms at a fixed rate of deoxygenation might also be explained as resulting from different rates of polymerization. Remember that the solubility of hemoglobin S decreases very rapidly at high fractional saturations and much more slowly at low fractional saturations (Fig 2 b). Consequently at a fixed deoxygenation rate dense cells become much more supersaturated and consequently polymerize with much shorter delay times than the light cells. As a result dense cells would be expected to contain many more polymer domains and to have a lumpy, granular appearance, as opposed to a classic sickle shape. Cell morphology is therefore expected to be highly correlated with intracellular concentration and cell density. The results of morphological studies on cells polymerized at different rates[151] and on density fractionated cells are consistent with this explanation.[85] The observation of smooth discocytes containing polymer in time-resolved electron microscope studies may represent the initial phase in the formation of a granular form.[152]

GELATION IN VIVO AND VASO-OCCLUSION

We now turn to the question of obstruction of blood flow in the microvasculature resulting from intracellular gelation. Vaso-occlusion is believed to be the cause of pain crises and of the widespread organ damage that contributes substantially to the morbidity and mortality of the disease. Because of the enormous complexity of this problem, the discussion must, of necessity, become much more qualitative and speculative than that which has been presented up to now. We shall see that there are suprisingly little hard data on some of the most basic questions about vaso-occlusion. Nevertheless we believe that a critical examination of this problem is necessary at this point to clarify the important issues and to point to areas where research is most needed. We first discuss gelation and vaso-occulsion, and in the next section we consider the response of the circulatory system to this abnormality.

To gain some perspective on the problem it is instructive to consider the various types of events that have been postulated to occur as a red cell travels through the circulation of an SS patient. In describing these events we shall equate sickling with intracellular gelation. Figure 8 shows a schematic summary. Cells containing no polymerized hemoglobin in the arterial circulation may pass through the microcirculation and return to the lungs without sickling, they may sickle in the veins, or they may sickle in the capillaries. The probability for each of these events will be determined by the delay time for intracellular gelation relative to the appropriate transit time.[7] If it is thermodynamically impossible for gelation to take place (ie, the intracellular concentration is always lower than the solubility so that at equilibrium no polymer can form) or if the delay time at venous oxygen pressures is longer than about 15 seconds, then sickling will not occur. If the delay time is between about one and 15 seconds, then the cell will sickle in the veins, and, if it is less than about one second, the cell will sickle within the capillaries. For cells that sickle within the capillaries a number of possibilities exist, ranging from no effect on its transit time to transient occlusion of the capillary or a more permanent blockage that ultimately results in destruction of the cell. For some cells the intracellular hemoglobin S concentration may be so high that the solubility is exceeded even at arterial oxygen pressures. These cells will still contain polymerized hemoglobin after oxygenation in the lungs. Upon deoxygenation further gelation will occur rapidly and without a delay time because nucleation of polymers is already complete.[7,22,77,136,137] Such cells could become stuck in the arterioles or capillaries or could experience a normal transit time through the microcirculation in spite of the decreased deformability.

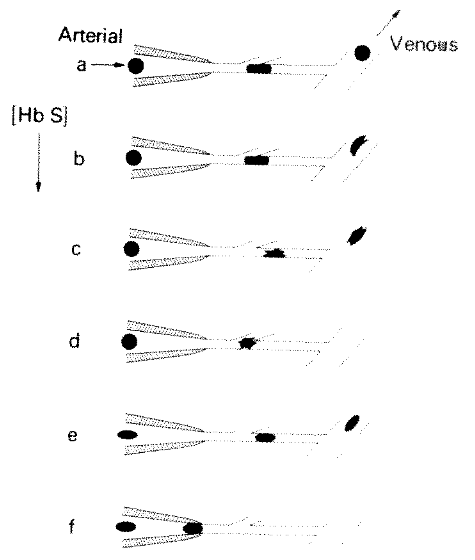

Fig 8. Possible events in the microcirculation of a patient with homozygous SS disease. A schematic of an arteriole, capillary, and venule is shown. In (a) a cell containing no polymer enters the capillary, deforms to squeeze through, and reaches the venule without polymerization occurring. In (b) the delay time is longer than the capillary transit time, but the cell sickles in the venule. In (c) the delay time is shorter than the capillary transit time, and the cell sickles within the capillary but escapes to the venule, while in (d) intracapillary sickling results in transient or permanent blockage. In (e) and (f), the cell, depicted as an irreversibly sickled cell, already contains polymerized hemoglobin in the arteriole and may pass through the capillary (e) or produce a transient or permanent occlusion (f).

Figure 8 points out one fundamental problem in describing the pathophysiology of sickle cell disease is to determine the relative probabilities for each of these events. These probabilities will depend on a number of factors, including the total intracellular hemoglobin concentration, the composition of the intracellular hemoglobin, the rate and extent of deoxygenation, and the various transit times involved. For unsickled cells entering the microcirculation, a long capillary transit time will increase the probability of the potentially vaso-occlusive events depicted in Fig 8 in two ways. First, it will permit increased oxygen extraction, which will shorten the delay time. Second, it will increase the probability that a cell with a given delay time will sickle within the capillary. For cells that either enter the microcirculation already sickled or become sickled in the microcirculation, there is a finite probability for occlusion of the small vessels. The duration of an occlusion may be sufficiently long to compromise the oxygen supply to the surrounding tissues and hence may alter the probabilities for sickling and consequent vaso-occlusion in nearby microvessels. This is a somewhat refined version of the "vicious cycle."[153] It is important to recognize that vaso-occlusion is a dynamic process in which the fraction of capillaries that are occluded depends on both the rates of occlusion and the rate of capillary reopening. Thus factors that influence the transit times and the duration of occlusions also play a critical role in the pathophysiology.[7]

With this brief heuristic description as a framework for subsequent discussion, we can now proceed to examine experimental results that help to establish the probabilities for the various events depicted in Fig 8. The most straightforward problem is the determination of the fraction of cells that are sickled in the arteries and the fraction that sickle as a result of deoxygenation in the microcirculation. Morphological examination of cells sampled from arterial blood suggests that the average fraction of sickled cells is about 10%.[78,154-156] This number is, unfortunately, only a rather crude estimate because it is possible that deformed cells such as granular discocytes have not been counted as sickled in some studies; moreover, some irreversibly sickled cells, which may frequently be a major contributor to this count,[156] may contain no polymer.[140] The values for reversibly sickled cells in different patients range from 1% to 16%, while total sickled cell counts range from 9% to 30%.[156] This variation presumably results from differences in the distribution of intracellular hemoglobin composition and concentration, as well as from differences in arterial oxygen saturation.

It would appear, then, that an average of about 90% of cells entering the peripheral circulation contain no polymer and hence would undergo gelation with a delay period if sufficiently deoxygenated. The morphological data suggest that about 20% of cells are sickled in the mixed venous return,[78,154-156] indicating that an additional 10% of cells have sickled as a result of passing through the microcirculation.* Any analysis based on studies of mixed arterial and venous blood is clearly somewhat oversimplified because oxygen extraction in the microcirculation of some tissues, such as in the coronary and hepatic circulations,[155] is considerably greater than average. As a result the cells in these organs will have much shorter delay times leading to a higher number of sickled cells in the veins.

These findings are consistent with existing information on in vitro delay times. Although the ideal in vitro experiment in which gelation and degelation are continuously monitored in individual red cells at physiologic rates of deoxygenation and oxygenation is not yet possible, the laser photolysis experiment[136,137] affords an informative preview of the results expected from such experiments. The *unsickling* curves obtained in these experiments (Fig 7) show that *at equilibrium* only about 5% of the cells contain polymerized hemoglobin S at an oxygen pressure of 85 torr, which is the average arterial value found in homozygous SS patients,[78] while over 90% of cells remain sickled at the average mixed venous pressure of 45 torr. In contrast, the *sickling* curves show that only about 5% of cells (overlapping significantly with the cells that were found to be sickled at 85 torr at equilibrium) are sickled after deoxygenation to venous oxygen pressures on physiologic time scales. These results indicate that the delay time is preventing more than 80% of cells from sickling in vivo in this homozygous SS patient. That is, for over 80% of cells gelation would occur if equilibrium were achieved, but the delay times are so long that these cells return to the lungs and are reoxygenated before any significant amount of polymer has formed. The fact that about 10% of cells already contain polymer in the arterial circulation does not substantially affect the fraction of sickled cells in the microcirculation, since if polymer were not present the delay times for the large majority of these cells would be less than the capillary transit time.† The enormous difference between the unsickling curve and the sickling curve in these experiments (Fig 7) graphically demonstrates the significance of the delay time for gelation in vivo and simultaneously shows that equilibrium data or data obtained in slow deoxygenation experiments are not at all representative of the in vivo situation in which the relevant time scale is seconds. Similar large differences are expected for oxygen binding and polymer fraction curves (Fig 2 e, 2 f and discussion below).

This analysis points to the critical need for obtaining much more data relevant to sickling in vivo. An accurate description would require direct measurements of the distribution of delay times at physiologic rates and extents of deoxygenation. It would also be desirable to have more precise data on the extent of sickling in arterial and venous samples using experimental methods that take into account the kinetics of sickling as well as the recently acquired information on the relation between cellular deformation and intracellular gelation.[136,137,140] Most of the data on morphological sickling were obtained before the gelation kinetics were described, and since then very little attention has been given to designing accurate morphological experiments on venous and arterial samples. Such experiments would require rapid fixation of cells and careful examination by scanning electron microscopy or high resolution optical microscopy. It would, of course, be preferable to develop rapid sampling techniques that could assess the extent of intracellular gelation, or at least the presence or absence of polymer, in individual cells from arterial and venous blood.

We next consider the question of occlusion of the microcirculation by sickled cells, which is clearly a central problem in understanding the pathogenesis. The results and calculations described above as well as data on red cell survival suggest that the densest cells are primarily responsible for occlusion of the microcirculation. These cells are predicted to have a much greater probability for the events in Fig 8 that could lead to vaso-occlusion. Because of the high intracellular hemoglobin S concentration, they have shorter delay times, or no delay times and therefore are more likely to sickle in the capillaries when deoxygenated.[6,7] In addition, if gelation occurs at any given oxygen pressure, the higher intracellular concentrations of polymerized hemoglobin (Fig 2 d) produces stiffer cells (ie, cells with lower static and dynamic deformabilities[140]). The half-life of dense cells (~ two days) is significantly shorter than that of randomly labeled cells from the same patient population (~ five days)[164] or the average half-life of cohort-labeled cells from a wider patient population (~ 17 days).[164-166] Experiments in which the density distribution of a population of labeled reticulocytes was followed over the cell life span also show that the dense cells are the last to appear in the labeled population.[164] These results show clearly that as cells age their density increases and that once they become dense they are removed from the circulation rather quickly.[164] The mechanism by which cells concentrate has been the subject of much recent work.[167] The major contribution appears to result from the loss of cell water associated with potassium loss, but there may also be some contribution from the loss of membrane surface area caused by the sickling-unsickling cycle. If it is assumed that all cells must become dense cells before they are removed from the circulation, then the fraction of dense cells is predicted to be equal to the ratio of the half-life of the dense cells to the mean half-life for new cells. The measured half-life of the dense cell fraction is roughly consistent with this prediction, since the densest 10% to 15% of the cell population has a half-life that is 0.1 to 0.2 times that of labeled reticulocytes.[164] Cells that have an abnormally low probability of sickling, such as low density F cells, are expected to have extremely long delay times even at venous saturations. Since these cells can only be sickled upon stasis or passage through tissues where deoxygenation is extreme, they will presumably concentrate much more slowly than cells having a higher probability of sickling. This protection of F cells results in a longer life span[1] and therefore an increased concentration of F cells in the fractions of intermediate density, which represent the oldest cells in the population, and a reduced concentration in the densest cell fraction.[87,164]

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*The estimate of 10% additional sickling in the microcirculation is only a very approximate number. Differences in this number are observed for different venous returns of the same patient and for the same venous return in different patients, but the tissue-to-tissue variation is generally smaller than the differences observed between patients.[78,155,156] Since cells are deoxygenated in the microcirculation within one to two seconds, significant variations may result from additional sickling during the time required to sample the cells from the veins and to fix them with glutaraldehyde and possibly some additional unsickling for cells sampled from the arteries.[157,158]

†Shearing forces are known to decrease the delay time by breaking polymers, producing new ends, and thereby increasing the rate of nucleation.[121,139,159,160] In this discussion we have assumed that there is no significant effect of shear on the in vivo delay time. While no direct experimental information on this point exists, two considerations suggest that the effect of shear on intracellular polymerization in vivo will be small. First, cells flowing in small tubes concentrate in the low-shear region near the center of the tube, while the high shear regions near the walls are preferentially occupied by plasma.[161,162] The shear field to which cells are exposed is thus very much smaller than the average field in the microvessels. Second, the high internal viscosity of even unpolymerized cells makes coupling of the external shear field to the inside of the cell inefficient, as evidenced by the absence of tank-treading behavior in low viscosity media.[163]

Of the vaso-occlusive events depicted in Fig 8, it is only possible to make even a crude quantitative estimate for the probability of occlusion followed by *destruction* of the cell. Based on a mean cell lifetime of 17 days and a circulation time of 15 seconds, an "average" red cell makes about 100,000 trips through the microcirculation before being removed. If we use the fraction of sickled cells as an estimate of the fraction of dense cells (~20%), the above argument suggests that if dense cells were removed from the circulation only by vaso-occlusive events, they would be trapped and destroyed once in about 20,000 trips through the microcirculation. However, hemolysis data indicate that about 60% to 70% of sickle cells are destroyed in the reticuloendothelial system,[166] so the probability for destructive vaso-occlusion is reduced to about one in 60,000 trips. While this probability appears, at first glance, to be extremely low, it is large enough to result in the steady-state blockage of a significant fraction of the total number of capillaries, since there are approximately 10^{13} circulating red cells and only about 10^{10} capillaries.[168] The exact fraction blocked is highly dependent on the duration of the destructive blockade, which, by this estimate, occurs in the average capillary about once every hour. If, for example, each blocked vessel remains occluded for one hour, the fraction blocked is 50%, but this value is reduced to 2% if the vessel is blocked for only one minute. This result clearly suggests that factors that influence the duration of a capillary blockade could play a critical role in determining the extent to which tissue oxygen supply is compromised.

It has been very difficult to obtain quantitative information on the frequency of the other events depicted in Fig 8. Recently a series of studies using an excised rat mesocecum preparation has begun to provide some interesting results. This preparation permits control over the tissue oxygen pressure and the perfusing pressure, allowing vascular resistance (defined as the ratio of the arteriovenous pressure difference divided by the venous outflow) changes to be measured quantitatively in both denervated[85,86] and innervated vascular beds.[169] Alternatively, trapping of cells can be measured by first perfusing the innervated bed and subsequently washing out the trapped cells by increasing the perfusion pressure or denervating the prepared bed.[169] These experiments make two important points. The first is that at venous Po_2 the fraction of capillaries blocked at steady state in this preparation can be as large as 80%. The second is that the ability of cells to block the microcirculation is correlated with their density, a finding that is consistent with the description of Fig 8.

These investigations provide the best opportunity to simulate the events occurring in the microcirculation of SS patients. In addition to measuring vascular resistance and cell trapping, cinematographic observations of these preparations permit determination of the sites at which blockage occurs. The limited information obtained so far has not established the relative importance of precapillary and intracapillary sites as the principal sites of occlusion.[170-172] It would be important to extend studies of the microvasculature to use preparations that permit deoxygenation in the tissue[170] to more closely simulate the in vivo situation. It will also be important to use these preparations to determine the factors that affect both the frequency and duration of occlusions, since they are equally important in determining the steady-state fraction of blocked capillaries.

GELATION AND OXYGEN DELIVERY

An important aspect of the pathophysiology of sickle cell disease is to understand how the circulatory system maintains adequate oxygen delivery in the face of anemia and vascular obstruction. In other severe anemias, for example, those arising from blood loss or iron deficiency, there are two primary compensation mechanisms. One is an increased blood flow through the tissues resulting from an expansion of the muscled arterioles.[173,174] The increased blood flow from this decreased peripheral resistance increases filling of the right atrium, thereby increasing the cardiac output.[175] The second is an increase in intracellular 2,3-DPG concentration, which lowers the oxygen affinity and facilitates oxygen unloading in the tissues.[25] As a result there is an abnormally low venous oxygen saturation at or near a normal oxygen pressure.[173,174,176-178] The increased blood flow through the lungs may also result in a decreased oxygen pressure and saturation in arterial blood[173,176,179] from the decreased efficiency of gas exchange,[180] making oxygen delivery less efficient.

In sickle cell disease the abnormalities are somewhat different. The cardiac output is higher than in anemias of comparable severity,[78,173,176,181] the oxygen pressure and saturation of arterial blood are lower,[78,154-156,182,183] and the oxygen pressure and saturation of venous blood are higher.[78,154,156] This decrease in arteriovenous saturation difference, particularly when compared to other anemic states, means that there is significant impairment of oxygen unloading to the tissues.[78] These differences between sickle cell disease and other anemias presumably result from the intracellular gelation and vascular obstruction that are unique to sickle cell disease.

The very low oxygen affinity of polymerized hemoglobin S explains the lower arterial oxygen saturation in sickle cell disease, compared with other anemias, and even the lower arterial oxygen pressure. Infarctive damage to lung tissue of SS patients could also decrease the efficiency of oxygen loading in the lungs, but this does not appear to be a major factor because a comparable degree of arterial unsaturation is observed in children in whom there is no other evidence of impaired lung function.[184,185] We have estimated earlier that about 20% of cells entering the lungs contain substantial amounts of polymerized hemoglobin, which would be expected to decrease both the rate and extent of oxygen binding to the sickled cells in the alveoli. The few in vitro experiments support this contention.[152,157,158,186] Slow depolymerization of the sickled cells after they leave the lungs could also contribute to the lower arterial oxygen tension by scavenging oxygen from the plasma and from the cells that contain no polymer.

In considering oxygen unloading in the tissues, the absence of polymerized hemoglobin in most cells because of the long delay times is an important consideration. The traditional approach has been to ignore this fact and to utilize oxygen dissociation curves measured in vitro over periods of minutes or longer where intracellular gelation is much more extensive than in the in vivo situation. Use of the in vitro curve has led to the conclusion that the large right shift in the equilibrium or quasi-equilibrium dissociation curves substantially compensates for the anemia.[173,187,188] This conclusion is misleading, since oxygen binding to approximately 80% of the cells should be similar to that found for other states of comparable anemia. The calculation in Fig 2 e shows that the in vivo oxygen unloading curve is predicted to be significantly less right shifted than the in vitro equilibrium curve. This smaller right shift is consistent with the observation that the fractional saturation of venous blood from sickle cell patients has near normal values, while the oxygen pressure is higher than in normal blood by about 5 torr.[78]

Perhaps the most puzzling circulatory abnormality is an increase in cardiac output that is larger than is found in anemias of comparable severity. The absence of a significant increase in arterial blood pressure[189] requires that the vascular resistance be *decreased* in direct proportion to the increase in cardiac output.[78] While decreased vascular resistance may appear paradoxical in the presence of capillary blockage,‡ this finding can be rationalized by the fact that the bulk of the peripheral resistance arises from the muscled arterioles and not the capillaries.[192] In severely anemic states the arterioles open to increase blood flow through the tissues, thereby compensating for the low hematocrit.[173,174] A similar response also appears to be the primary mechanism of compensation for the reduced hematocrit in SS disease, but the peripheral resistance is decreased to a significantly greater extent than in other anemias of comparable severity.[78,173] The opening of the muscled arterioles must therefore increase the fraction of capillaries in the tissue bed that are perfused to above normal, even in the presence of blockage, if there is sufficient capillary reserve.

In addition to increasing the number of perfused capillaries, there is evidence that opening of the muscled arterioles increases the pressure drop across the capillaries, thereby increasing the rate at which red cells traverse the capillaries.[192-194] A recent study in which laser Doppler velocimetry was used to measure capillary flow in the forearm skin of SS patients showed that the average rate of *red cell* flow was close to normal.[195] This result implies that the ~40% decrease in hematocrit is almost exactly compensated by a combination of an increase in the number of perfused capillaries and an increase in capillary flow rate. By decreasing the time available for the equilibration of the red cell with the oxygen tension of the capillary wall, an increased capillary flow rate would be expected to decrease oxygen unloading. In spite of this effect a decreased capillary transit time could benefit the patient by decreasing the rate of capillary blockage. The decreased transit time would not only increase the delay time by increasing the final fractional saturation of hemoglobin but it would also decrease the time during which a cell is at risk from sickling within the microcirculation. Such a mechanism provides an attractive explanation for the decreased arteriovenous oxygen saturation difference in SS patients. A frequently invoked explanation for the low arteriovenous saturation difference is shunting through large vessels.[154]

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‡There is no direct measurement of the fraction of occluded capillaries in any tissue in sickle cell disease. However, a tentative estimate for muscle can be extracted from data on the exercise tolerance of sickle cell patients.[190] In these studies patients were subjected to increasing work loads, and the lactic acid level in the blood was monitored. The work load at which lactate began to increase is defined as the anaerobic threshold. An extension of the Krough model for oxygen delivery to tissues predicts that the work output at this point is nearly directly proportional to the density of perfused capillaries.[191] If it is assumed that the muscle is maximally perfused and that capillary densities in the muscle of SS patients are normal, then the fraction of blocked capillaries can be estimated from the anaerobic threshold to be about 0.4. This fraction decreases to about 0.1 to 0.2 when the fraction of SS cells is decreased to about 50% by exchange transfusion.[190]

While this mechanism might account for the unexpectedly high venous saturation in specific tissues, it cannot explain the fact that similar results are found for vessels such as the femoral vein.[78] The femoral vein primarily drains muscle beds in which there is anatomic evidence that significant shunting is impossible.[196]

Finally, we should point out that there has been only one attempt to quantitatively evaluate the response of the complete circulatory system to the altered properties of SS blood.[197] This study used an established model for microcirculatory control[198] to calculate the changes in peripheral resistance blood flow, and capillary oxygenation.[197] This model incorporates approximate but realistic descriptions of oxygen supply and consumption in the tissues as well as local feedback control of both the arteriolar resistance and capillary density to regulate the tissue oxygen pressure. When anemia is simulated by reducing the hematocrit, the model predicts a compensatory decrease in peripheral resistance and increased blood flow. If, however, the quasi-equilibrium increase in viscosity and reduced equilibrium affinity of SS blood are also introduced, the model predicts a capillary resistance that is about 1.4 times normal and blood flow that is about 80% of normal. The calculated effects are in striking contrast to the observed *decrease* in peripheral resistance and *increase* in blood flow.[78,181] The discrepancy presumably results in part from the incorrect assumption of equilibrium oxygen unloading and viscosity changes made in carrying out these calculations. If the kinetics of intracellular gelation were to be incorporated into this model (Fig 2 e), the predicted effects would be closer to the observed.

VARIATIONS IN CLINICAL SEVERITY

It is now widely recognized that there are large differences in clinical severity among patients with homozygous SS disease, some patients having only the mild symptoms associated with a chronic hemolytic anemia, others suffering from repeated painful episodes and severe organ damage.[24,25,199,200] The reasons for this broad spectrum of clinical manifestations are not at all clear, and it is one of the major areas of current research. To discuss the role of gelation in producing differences in clinical severity among homozygous SS patients, it is useful to briefly summarize the most important results of the preceding discussion.

The picture that emerges is a dynamic one in which a balance between the rate of obstruction and reopening of capillaries results in a steady state in which a certain fraction of capillaries is blocked in each tissue. This balance may be very delicate, with small changes in either the rate of obstruction or reopening capable of significantly altering the fraction of occluded capillaries. Any increase in this fraction, particularly in tissues with inadequate capillary reserve, could result in irreversible hypoxic damage and may be the cause of pain crises. While almost nothing is known about the opening of occluded capillaries, we are beginning to understand the mechanisms that control the rate of capillary obstruction. This rate must depend, at least in part, on the fraction of sickled cells in the microcirculation, which is determined by the times required for intracellular gelation relative to the transit times.[6,7] Factors that favor gelation can increase the steady state number of obstructed capillaries by decreasing the delay time, thereby increasing the fraction of cells that sickle within the microcirculation and the rate of obstruction. An increased extent of polymerization in a sickled cell could also increase the probability of an occlusion because of the decrease in deformability.[140] Because the delay time is so much more sensitive to changes in physiologic variables than the extent of polymerization (compare, eg, Figs 2 d and 3), it is most probably the dominant factor in determining changes in the rate of capillary obstruction. Simultaneous small changes in a number of physiologic variables could result in a sufficient change in the distribution of delay times to produce the fluctuation in the fraction of blocked capillaries that precipitates a pain crisis. In this way the sensitivity of the delay time could account for the episodic nature of crises.

Another mechanism for increasing the rate of obstruction is to increase the transit time in the microcirculation, which increases the probability of sickling. In this way factors that slow down cells can also affect the rate of obstruction. The only such factor that has been identified so far is the adherence of cells to the vascular endothelium.[201-204] In addition to capillary blockage, other events influence these probabilities by altering the characteristics of the cell population. For example, cells that normally would return to the lungs may sickle in the venous return, particularly in tissues in which the residence times in the veins are long, resulting in an increase in intracellular concentration and therefore a decreased delay time in subsequent trips through the microcirculation.

This picture immediately raises the question of how much of the variation in clinical severity in homozygous SS disease can be explained by variations in intracellular gelation and how much must be attributed to variations in circulatory dynamics. Under the category of intracellular gelation are included the effects of intracellular hemoglobin concentration and composition, arising either from genetic variations or from cell aging. To begin, let us consider the relation between gelation times and clinical severity among the various sickling disorders, where there are easily measurable differences in both gelation and standard hematologic parameters. Figure 9 shows the effect of hemoglobins A, C, and F on the delay time and a comparison of the distribution of delay times at zero saturation for the three most common syndromes: homozygous SS disease, SC disease, and sickle trait. SC disease is generally a much milder sickling disorder than is SS disease, while sickle trait is totally benign.[24,25] The delay times for SC cells are considerably longer than those for SS cells, indicating that many fewer cells sickle in vivo. For sickle trait cells the delay times even at zero saturation are all longer than about one second, indicating that even under totally anoxic conditions cells would escape the microcirculation before polymerization has begun. With the possible exceptions of the hypertonic renal medulla, it would appear that sickle trait cells never sickle in vivo, explaining the lack of any clinical manifestations.

The reasons for the increased sickling of SC cells compared to sickle trait cells are quite interesting. Little or no difference is observed in the gelling properties of hemoglobin S + C mixtures and S + A mixtures. A careful comparison has shown that there are no significant differences in either

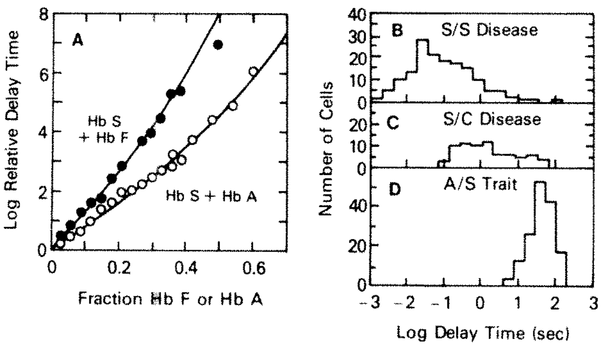

Fig 9. Effect of non-S hemoglobins on gelation delay times in solutions and cells. (A) Logarithm of the ratio of the delay time of the mixture to the delay time of pure deoxyhemoglobin S at the same total hemoglobin concentration.[57] The effect of hemoglobin C on the delay time is identical to that of hemoglobin A.[62] (B, C, D) Distribution of delay times at zero saturation for cells from a patient with homozygous SS disease (B), hemoglobin SC disease (C), and sickle trait (D) at 37°C. The data in (B) and (C) are taken from Coletta et al,[135] while the data in (D) is from Zarkowsky and Hochmuth[143] after using the temperature dependence of the median delay time to correct the data to 37°C.

the delay times[62] or solubilities.[62,63] The principal reason for the increased sickling of SC cells is that they contain a higher hemoglobin S concentration than sickle trait cells. This increase results from two effects.[62] First, there is a greater fraction of hemoglobin S in SC cells (50/50 S/C) than in sickle trait cells (40/60 to 30/70 S/A). The reduction in the fraction of hemoglobin S in sickle trait cells is caused by a decreased rate of association of α chains to β^S chains relative to β^A chains during the tetramer assembly process.[205,206] When the concentration of α chains is reduced because of coexisting α thalassemia, this competition is enhanced and a disproportionately larger fraction of β^A-containing tetramers are formed. Second, the total intracellular hemoglobin concentration is higher in SC cells.[62,84] Since reticulocytes have nearly the same density distribution as the average cell population, the red cells must emerge from the marrow more concentrated. The reasons for this are not yet completely understood, but it has been suggested that the binding of hemoglobin C to the red cell membrane induces a potassium and water efflux.[207]

Hemoglobin F also has a marked effect on gelation. This is clinically most evident in the uncommon double heterozygous condition of hemoglobin S with pancellular hereditary persistence of fetal hemoglobin, which may be asymptomatic. In this condition hemoglobin F is more evenly distributed, and most cells contain a substantial amount of hemoglobin F (up to 35%).[24] This mixture has gelling properties in vitro that are similar to the 40/60 Hb S + A mixture found in sickle cell trait (Fig 9)[37,57] and would therefore be predicted to have a very mild or asymptomatic clinical course. In homozygous SS disease there is a variable increase in hemoglobin F that results from two factors: an increased production of F reticulocytes and preferential survival of F cells.[81] At hemoglobin F levels above 20%, corresponding to about 60% F cells, there may be some amelioration of the disease, but below 20% there appears to be no significant effect.[201,208]

Because of the tremendous sensitivity of the delay time to the total hemoglobin concentration, it has been suggested that the clinical severity of homozygous SS disease may be improved by a small dilution of the intracellular hemoglobin.[6,7] The increase in the delay time resulting from a decrease in the intracellular concentration would allow more cells to escape the microcirculation before gelation has begun.[6,7] To estimate the effect of concentration on the delay time we use a 15th power inverse concentration dependence, since this is the concentration dependence found for delay times of about one second (Fig 3). The decrease in MCHC from 32 g/dL to 30 g/dL associated with the coexistence of α thalassemia, in which two of the four α genes are deleted $(-\alpha/-\alpha)$,[239] produces an almost three-fold increase in the delay time for the "average cell." The result is increased red cell survival[87,210] and an indication of fewer episodes of the acute chest syndrome and leg ulceration.[211,212] Also, in SS disease there may be an increased frequency of the α gene deletion with age, suggesting that a decreased total intracellular hemoglobin concentration is associated with a longer life expectancy.[213] In HbS-β^0-thalassemia there is a similar decrease in MCHC, and the clinical course relative to SS disease is "milder in many features."[24]

Thus far we have seen that for genetically different sickling disorders there is a good correlation between intracellular gelation in vitro, for solutions having compositions of the average cell, and disease severity for the average patient.§ To investigate the role of clinical diversity one would ideally want to know at least the distribution of intracellular delay times for patients from a clinically well-characterized population. No such data are yet available. An efficient but limited method of examining distributions of intracellular gelation is to measure density distributions, since the density is proportional to the total intracellular hemoglobin concentrations. Differences in intracellular solvent conditions of pH, 2,3-DPG concentration, etc, are expected to have a much smaller effect on gelation than differences in intracellular hemoglobin concentrations. Consequently the distribution is expected to reflect the distribution of intracellular delay times, except for the effect of F cells.

The only study carried out so far is one in which cell density distributions were compared with the incidence of painful crisis.[214] No correlation was found between the fraction of cells in the highest density range and crisis frequency. This result was interpreted as evidence that the greater probability of intravascular sickling is not the principal cause of increased crisis frequency, but that variations in the anatomy and dynamic properties of the microcirculation are responsible for differences among patients. As pointed out earlier, one factor that could be important in determining transit times in the microcirculation is adherence to the vascular endothelium. A strong correlation has in fact been found between overall clinical severity and the tendency of the red cells to adhere to vascular endothelium in in vitro experiments.[201-203] The severity score used in this study included evidence for organ damage resulting from microvascular occlusions as well as the frequency of pain crises. In this same study there was no correlation between severity and hemoglobin F levels or irreversibly sickled cells, which are known to correlate with the fraction of dense cells.[82]

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§Correlations between gelation and both overall clinical severity and degree of anemia have also been obtained using the in vitro fraction polymerized *at equilibrium* as a measure of gelation in vivo.[209] Although the equilibrium fraction polymerized in vitro is not relevant to the in vivo situation as discussed earlier, these correlations give a very similar result[209] because of the close correlation between the kinetic and equilibrium properties of gelation.[36]

THE PROBLEM OF INHIBITING GELATION IN PATIENTS

The strong correlation between gelation and severity for the "average" patient with the various sickling disorders clearly indicates that inhibition of gelation should result in amelioration of the disease. The data on hemoglobin mixtures shows that it will not be necessary to completely inhibit gelation (ie, increase the solubility such that it equals or exceeds the total intracellular hemoglobin concentrations at all oxygen pressures) but that a therapeutic effect should result from sufficiently increasing the delay time to allow more cells to escape the microcirculation and be reoxygenated in the lungs before gelation has begun.[6,7,57,98] In this way there should be a reduction in the rate of production of dehydrated, rapidly polymerizing cells, which have been generally assumed to be the subpopulation of cells most responsible for initiating vaso-occlusion.[5] To give this concept a quantitative basis we may ask: how much must gelation be inhibited to obtain a specified therapeutic effect in patients? An approximate answer to this question can be obtained from the correlation between in vitro delay times or solubilities in solutions of deoxyhemoglobin mixtures having the compositions found in various sickling disorders and their "average" clinical course.[57]

The data for this comparison are found in Fig 9, and Table 1 shows the increase in delay time and solubility relative to pure deoxyhemoglobin S for solutions having the hemoglobin composition found in sickle-β^+-thalassemia, sickle cell disease with hereditary persistence of fetal hemoglobin, and sickle trait. The results in Table 1 establish a set of criteria for obtaining a specified therapeutic effect. They suggest that the threshold for obtaining a therapeutic effect in SS disease would result from a method that produces an increase in the in vitro delay time of about a factor of 100 (corresponding to a solubility ratio of about 1.2), which is the increase found for solutions having the hemoglobin composition of sickle-β^+-thalassemia; an increase of about 10^3 to 10^5 (solubility ratio of about 1.3) should produce a major therapeutic effect; and a 10^6 to 10^8-fold increase in the in vitro delay time (solubility ratio of about 1.5 to 1.6), found for solutions with the composition of sickle trait cells, is predicted to result in a "cure."

Table 1. Clinical Course, Gelation Delay Time and Requirements for Therapy

Disorder	S/β^+-Thalassemia	S/HPHF	A/S Trait
Clinical course relative to S/S disease	Less severe	Much less severe	No disease
Red cell composition*			
% Hb A	20-30	0	60-75
% Hb F	0	20-35	0
% Hb S	80-70	80-65	40-25
Log delay-time ratio†	1.5-2.5	2.5-5.0	6.0-8.0
Solubility ratio‡	1.1-1.2	1.2-1.35	1.45-1.65
Therapy requirements			
Percent saturation of inhibitory site§	20-40	40-55	≥65
Decrease in intracellular concentration (g/dL)‖	3-5	5-9	≥11

*The data are from Serjeant.[24] For S/β^+-thalassemia this is the composition of the non-F cells.

†This is the ratio of the delay time for the mixture to the delay time for pure deoxyhemoglobin S at the same total hemoglobin concentration and is obtained from the data in Fig 9. These ratios are for subphysiologic concentrations using the temperature-jump technique for measuring delay times. For physiologic concentrations where the dependence of the delay time on supersaturation is smaller, these ratios are expected to be smaller, as is found with intact cells (see Fig 9b to d).

‡This is the ratio of the solubility for the mixture to the solubility for pure deoxyhemoglobin S in the limit of no polymerized hemoglobin (from Eaton and Hofrichter[26]).

§This is the fractional saturation of an ideal inhibitory site, ie, one that completely prevents polymerization, required to produce the delay time increase for pure deoxyhemoglobin S.

‖This is the required decrease in intracellular concentration, assuming an intracellular hemoglobin S concentration of 34 g/dL in the cells entering the circulation in S/S disease.

Adapted from Sunshine et al.[57]

With these estimates we can examine the potential utility of the various strategies that have been proposed to inhibit gelation in patients. Four different approaches have been explored or considered in some detail: (1) blocking intermolecular contact formation in the polymer, (2) raising the oxygen affinity, (3) decreasing the total intracellular hemoglobin concentration, and (4) promoting fetal hemoglobin production. The oldest idea is to develop a competitive or covalent inhibitor that would bind stereospecifically to hemoglobin S and interfere with polymer formation. Two general types of mechanisms have been considered. In one the "drug" acts directly by binding to an intermolecular contact site in the polymer, thereby competitively inhibiting polymerization. In the other the "drug" inhibits polymerization indirectly by changing the conformation at the intermolecular contact site so that it no longer "fits" into the polymer. The direct approach to inhibiting gelation poses a number of problems. Unlike an enzyme, where a substrate analogue can be a powerful inhibitor of catalysis by binding to the active site, none of the known intermolecular contact sites provide such a target. There are no clefts, grooves, or other obvious structural features that can be used to design molecules with complementary structures that might bind to hemoglobin S with high specificity. Examination of the intermolecular contacts also gives no real clues. This result might have been anticipated because the interactions between molecules in the polymer are weak. One approach would be to determine the structure of hemoglobin-antibody complexes in which polymer contacts are the antigenic determinant. Since hapten-antibody interactions are generally much stronger, the antibody-binding site would be expected to have the structural features of a very effective inhibitor and hence could serve as a model for the ambitious organic chemist attempting to construct molecules that cover the contact sites.

A natural target on the hemoglobin molecule for attack by the indirect mechanism is the pocket between the β subunits, which constitutes a specific, relatively high-affinity binding site for 2,3-DPG. For example, bifunctional aspirin derivatives have been described that crosslink the β subunits by covalently binding to opposite β82 lysines.[215] Analysis of the three-dimensional structure of the complex by x-ray crystallography shows that this modification causes a shift in residues of the F-helix that are part of the acceptor site for the β6 contact region, explaining the very large increase in solubility (solubility ratios up 1.5).[19] Although these particular inhibitors may not turn out to be therapeutically useful, this and other recent studies[20,21] demonstrate the power and feasibility of using x-ray crystallography to understand the mechanism of action of inhibitors and to design more effective ones. Most studies of inhibitors of gelation have not taken such a "rational" approach. Nevertheless a number of effective inhibitors have been found, although none has been developed to the point of being a serious candidate for use in patients.[25,216-220]

A second, more speculative strategy for inhibiting gelation is to increase oxygen affinity by shifting the allosteric equilibrium toward the R structure. At any given oxygen pressure there will be a lower concentration of molecules in the T quaternary structure and therefore a decreased tendency to polymerize. Calculations based on the effect of saturation on gelation suggest that therapeutically useful effects might result, although homeostatic responses that maintain oxygen delivery could buffer the inhibitory effect.[57] One interesting way of shifting the allosteric equilibrium toward R and one that would require much lower doses of a drug than directly attacking the hemoglobin molecule would be to inhibit 2,3-DPG synthesis.[57] An additional beneficial effect would result from the fact that 2,3-DPG promotes gelation of T-state molecules.[39,42] It will be important to evaluate the effect of an increase in oxygen affinity in some detail because many inhibitors of deoxyhemoglobin S gelation also increase oxygen affinity.

The third strategy is to decrease the intracellular hemoglobin concentration, an idea directly generated from the kinetic studies.[6,7] This approach takes advantage of the enormous concentration dependence of the delay time. There are two obvious ways that could, in principle, be used to decrease the total intracellular hemoglobin concentration. One is to permanently increase the red cell volume, and the other is to reduce hemoglobin biosynthesis without a decrease in red cell volume, for example by slowly introducing iron deficiency.[7] There are some clinical data to suggest that concomitant iron deficiency is in fact beneficial.[221] The idea of swelling red cells has been tested in a preliminary way. A combination of sodium restriction, high fluid intake, and the use of an antidiuretic reduced the serum sodium to 120 to 125 mg/dL, which resulted in a 2 to 3 g/dL decrease in the MCHC.[9,222] Both the frequency and duration of painful crises appeared to be reduced. Although this study was quite limited, involving only three patients who served as their own controls, it suggests that small reductions in intracellular hemoglobin concentration may indeed have a therapeutic effect, as predicted from the in vitro gelation studies.[7] Another approach to swelling red cells in patients has been to alter the ion transport properties of the red cell membranes so as to affect a net water influx. Several agents have been described.[8,10-12,223,224] Of these the most extensively studied is ceteidil,[8,10,223,225] which may be effective in directly retarding the dehydration that produces the rapidly polymerizing dense cells as a result of sickling-unsickling cycles.[223] In a placebo-controlled, double blind study ceteidil had some effect in reducing the severity and duration of pain crises,[226] but there is yet no information on its effectiveness in decreasing crisis frequency or organ damage.

The fourth strategy for inhibiting gelation in patients is to stimulate the production of γ globin. As discussed earlier, the inhibitory effect results from the inability of the $\alpha_2\gamma_2$ or $\alpha_2\gamma\beta^S$ tetramers to copolymerize with $\alpha_2\beta^S_2$.[37] If γ chains are exchanged for β^S chains in all cells, then some therapeutic effect is expected with hemoglobin F levels of about 10% to 15% (Fig 9 and Table 1). If hemoglobin F is heterogeneously distributed, clinical data from Saudi Arabians, where sickle cell disease is milder, suggest that amelioration would result if the percentage of F reticulocytes exceeds 20%, which results in a steady-state level of about 60% F cells and 20% hemoglobin F.[227] Data on American blacks suggest that at hemoglobin F levels above 10% there is a decreased probability of major organ failure, while the threshold for a decrease in crisis frequency is about 20%.[208] Although the molecular mechanism is not at all well understood, significant stimulation of F reticulocyte production has been achieved in SS patients with two drugs: 5-azacytidine[3,4,228] and hydroxyurea.[5,229,230] With 5-azacytidine hemoglobin F levels of 12% and 20% were achieved in two patients treated for more than 100 days, and there was a concomitant decrease in pain crises.[228]

The preceding analysis indicates that there is cause for optimism, as there are several totally independent and viable approaches to the therapy of sickle cell disease. Too frequently a single approach has been criticized as not being useful because by itself it does not produce a dramatic effect in patients. There is, of course, no reason why a specific treatment for sickle cell disease could not consist of the use of several drugs simultaneously, each inhibiting gelation by a different mechanism and at nontoxic doses that would produce only a small effect if given alone.

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A Glycoprotein Inhibitor of In Vitro Granulopoiesis Associated With AIDS

By Ira Z. Leiderman, Michael L. Greenberg, Bernard R. Adelsberg, and Frederick P. Siegal

Patients infected with the human immunodeficiency virus (HIV) often present with neutropenia. To elucidate the mechanism(s) of this HIV-related neutropenia, we assessed the proliferative capacity of the granulocyte-macrophage progenitor cell (CFU-GM) from the bone marrow (BM) of 78 patients within the AIDS spectrum manifesting symptoms or signs related to HIV infection. Of these, 70 had a significant deficit in the growth of this committed progenitor when compared with normal controls ($P < .01$). Further analysis revealed that the nucleated bone marrow cells from AIDS and AIDS-related complex (ARC) patients inhibited the growth of CFU-GMs from normal individuals when cocultured in agar ($P < .001$). Control CFU-GMs were also inhibited when they were cultured over feeder layers containing patients' BM cells ($P < .001$). Conditioned media obtained from the liquid culture of patients' BM cells did not inhibit normal control CFU-GM growth to a degree different from that of the cells themselves ($P > .4$). Analysis of these conditioned media by polyacrylamide gel electrophoresis (PAGE) revealed a unique glycoprotein (gp) with a mol wt of 84 kd. Further studies revealed that this gp possessed the inhibitory activity. These data suggest that this gp may be an important factor in HIV-related neutropenia. The presence of gp84 was independent of drugs administered to the patients.

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LEUKOPENIA is frequently associated with human immunodeficiency virus (HIV) infection. Although lymphopenia, primarily due to a decrease in the absolute number of the T helper/inducer ($CD4^+$) subpopulation, has been emphasized in the literature,[1-3] neutropenia is seen in ~40% of patients who develop symptoms of HIV infection.[4,5] In addition, these patients apparently have a greater than normal tendency to become neutropenic after exposure to trimethoprim-sulfamethoxazole, an antimicrobial drug combination known to cause transient neutropenia in other patients.[6,7] The depletion of the helper/inducer T cell population has been attributed to cellular infection with HIV.[8] The CD4 molecule has been identified as a receptor for the virus, and its subsequent cytolytic activity depletes that cell population.[9-12] This mechanism has also been hypothesized for the depletion of $CD4^+$ monocytes.[13] Because the CD4 molecule has not been demonstrated on neutrophils, such a mechanism would be unlikely for the observed neutropenia.

To determine if the HIV-related neutropenia has a similar decrease in the growth of CFU-GMs, we cultured bone marrow (BM) cells from 78 patients within the AIDS spectrum manifesting symptoms or signs related to HIV infection for the preferential growth of this progenitor cell. These patients had decreased proliferation of their CFU-GMs, and their BM cells inhibited normal control CFU-GMs. Further studies revealed that a glycoprotein (gp) inhibitor of normal CFU-GM growth is recovered from the supernatants of liquid cultures of BM cells from HIV-infected individuals.

MATERIALS AND METHODS

Subjects. Fifty-three patients met the Centers for Disease Control's (CDC) case definition for AIDS[14]: 38 homosexual males, 13 intravenous (IV) drug abusers (IVDA) (9 males, 4 females), 1 male hemophiliac, and 1 female sexual contact of an IVDA. Twenty patients defined as having AIDS-related complex (ARC) had: (a) A decreased ratio (≤ 1.0) of helper/suppressor ($CD4^+/CD8^+$) T lymphocytes; (b) decreased in vitro lymphocyte blastogenic response to mitogens (phytohemagglutinin, PHA, concanavalin-A, and pokeweed mitogen, PWM); and (c) at least one of the following symptoms—fever of undetermined origin, nocturnal diaphoresis, lymphadenopathy, wasting, diarrhea, leukopenia, or oral candidiasis (12 homosexual males, 4 male and 4 female IVDA). Five homosexual males were classified as having persistent generalized lymphadenopathy (PGL); these patients also had a decreased ratio (≤ 1.0) of helper/suppressor T lymphocytes and lymphadenopathy at ≥ 2 extrainguinal sites but no other AIDS-defining symptoms.

Twenty-five healthy male and female medical personnel who denied any risk for developing AIDS were studied as normal controls. Informed, written consent was obtained from all subjects, and the project was approved by the institutional review board of the Mount Sinai School of Medicine (CUNY).

All subjects were screened for antibodies to HIV by an enzyme-immunoassay (lymphadenopathy-associated virus, LAV-EIA; Genetic Systems, Seattle). All subjects except the controls were repeatedly reactive in this assay, which has been shown to correlate completely with Western blot positivity and radioimmunoprecipitation with ^{35}S-cysteine–labeled HIV.[15]

Bone marrow and blood collection. BM was aspirated from the posterior iliac crest into a sterile plastic syringe containing 50 U preservative-free heparin (Weddel Pharmaceuticals, London) diluted in 1.0 mL McCoy's 5a tissue culture medium (GIBCO, Grand Island, NY). Peripheral blood (PB) for CFU-GM cultures was similarly collected.

Serum preparation. Venous blood samples collected into sterile evacuated tubes (Vacutainer, Becton Dickinson, Rutherford, NJ)

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were allowed to clot at 4°C. The samples were centrifuged at 2,000 g for 10 minutes at 4°C, and the serum was collected and frozen at −70°C until used.

Cell separation. The anticoagulated BM was transferred from the syringe to a sterile polystyrene centrifuge tube (Corning, Corning, NY) and centrifuged at 250 g for 10 minutes at ambient temperature (22 ± 2°C). The buffy coat was aspirated and diluted with 2.5 mL McCoy's 5a medium. A nucleated cell count was performed manually, and the cell suspension was diluted to the appropriate concentration with McCoy's 5a.

Anticoagulated PB was transferred to a sterile disposable centrifuge tube (Corning) and diluted with an equal volume of sterile Hank's balanced salt solution (HBSS, GIBCO). Ten milliliters of a Ficoll-Hypaque solution (sp gr = 1.077) (LSM, Litton Bionetics, Kensington, MD) was layered under 40 mL diluted blood. The gradient was centrifuged at 250 g for 20 minutes at 4°C. The mononuclear cells (PB-MNCs) were aspirated from the Ficoll-Hypaque/plasma interface and washed twice with HBSS and were then diluted with 2.5 mL McCoy's 5a to the appropriate concentration.

Granulocyte-macrophage progenitor cell culture. A short-term, semisolid culture was used, similar to the system originally described by Pike and Robinson.[16] Cells in McCoy's 5a were cultured in a final concentration of 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 10% giant cell tumor-conditioned medium (GCT-CM; GIBCO) as a source of colony-stimulating factor (CSF),[17] and 2 mmol/L L-glutamine and 1% antibiotic solution (100X PSF; Gibco). Warm bacto agar (Difco, Detroit) was added to a final concentration of 0.3%, and the suspension was well mixed. Then, 1.0 mL was dispensed into each of four 35 × 10-mm tissue culture dishes (Corning) and allowed to solidify. Final cell concentrations were 0.2×10^6/mL for BM cells and 1.0×10^6/mL for PB-MNCs. The cultures were incubated in an atmosphere of 5.0% CO_2/95% air at 37°C for 10 days. Colonies were scored as ≥40 cells/aggregate. All cultures were scored in triplicate on day 10. These final concentrations of BM nucleated cells and PB-MNCs were chosen because they produced approximately the same CFU-GM growth in healthy controls.[18]

Conditioned medium. Nucleated BM cells from patients and controls were prepared as described above. The nucleated cells were diluted to a concentration of 1.0×10^6/mL in McCoy's 5a supplemented with 20% heat-inactivated FBS. The cell suspension was dispensed into 25 cm^2 or 75 cm^2 tissue culture flasks (Corning), depending on the final volume. The cells were cultured in an atmosphere of 5% CO_2/95% air at 37°C for 7 days. The CM was harvested by double centrifugation to remove all cells. The cell-free CM was frozen at −20°C until used.

Feeder layers. Feeder layers (FLs) were prepared from BM cells or CM. The cells or CM was diluted in McCoy's 5a and supplemented with a final concentration of 20% heat-inactivated FBS, 2 mmol/L L-glutamine, and 1.0% antibiotic solution (100X PSF). Warm bacto agar was added to a final concentration of 0.5%. After thorough mixing, 1.0 mL of suspension was dispensed into 35 × 10-mm tissue culture dishes and allowed to solidify. FLs were overlayered with normal target cells in 0.3% agar as described above. The amount of CM, or CM precipitate or PAGE eluate (below), used in these cultures was the amount calculated to be produced by 0.2×10^6 BM cells.

Ammonium sulfate precipitation. CM was precipitated with saturated ammonium sulfate, which was added dropwise to an equal volume of CM with continuous stirring at 4°C. The stirring was continued for 2 hours. The CM/salt solution was then allowed to sit undisturbed overnight at 4°C. The precipitate was then pelleted by centrifugation at 15,000 g for 20 minutes at 4°C. The supernatant was saved, and the pelleted precipitate was resuspended in McCoy's 5a to a volume equal to the starting volume of the CM. Both the resuspended precipitate and the supernatant were dialyzed against 0.015 mol/L phosphate-buffered saline (PBS) at 4°C. Three buffer changes were made over 48 hours of dialysis. The dialyzed material was then removed and stored at −20°C until used.

PAGE. Ammonium sulfate precipitates of the CM were diluted 1:5 with sample buffer pH 6.8 (0.5 mol/L Tris-Cl, 1.25% glycerol, 0.05% bromophenol blue) and then run on 7.5% native polyacrylamide gels (PAGE); mol wt was determined in 7.5% gels containing 1% sodium dodecyl sulfate (SDS).[19] All gels were run at a constant current as a discontinuous system with the 7.5% running gel at pH 8.8, the 3% stacking gel at pH 6.8, and the Tris-glycine running buffer at pH 8.8. Proteins running into the gel were visualized by silver staining.[20] Glycoprotein (gp) bands were visualized by periodic acid-Schiff (PAS) staining.

Elution of proteins from PAGE. Proteins were eluted from the gel slices in an ISCO sample concentrator (Model 1750, ISCO, Lincoln, NB). In brief, lanes containing CM from both patients and controls were cut from the gels, and the individual lanes were sectioned. The gel sections were placed in individual sample cups to which 10 mL Tris-glycine buffer (pH 8.8) was added. Elution of the proteins was achieved at 3 W constant power for 2 hours. At that time, the protein/buffer solution was removed and dialyzed as described above. After dialysis, the protein concentration was determined by the method of Bradford.[21] This dilute protein solution was concentrated by centrifugation through a Centricon-10 microconcentrator (Amicon, Danvers, MA), and the final protein concentration was redetermined. Inhibitory activity was assayed by adding the concentrated eluate to the FL in place of the CM.

Western blot. The Western blot technique was adapted from the EIA described by Tsang and colleagues.[22] Antigen is run into a native or SDS-PAGE. A nitrocellulose membrane (BA-85; Shleicher and Schuell, Keene, NH) is placed over the gel, and the gel/nitrocellulose is sandwiched between two plastic pads. The sandwich is then immersed into cold Tris-glycine/methanol buffer and electrophoresed with constant voltage for 12 to 14 hours at 4°C. The nitrocellulose membrane is then incubated with the first antibody, rewashed, and then reincubated with a horseradish peroxidase-conjugated second antibody. Diaminobenzidine is then added, and any positive bands are visualized.

Statistics. Data are presented as the mean ±1 SD. Statistical significance of the differences between groups was determined by Student's *t* test.

RESULTS

Leukocyte counts. WBC and neutrophil counts for all patients studied are summarized in Table 1. The normal WBC count for normal adults in this laboratory is 4.8 to 10.8×10^6/mL, and the normal neutrophil count is 1.9 to 8.0×10^6/mL. The mean ± SD WBC count of all AIDS patients studied was $3.3 \pm 1.8 \times 10^6$/mL, for ARC patients $4.5 \pm 1.7 \times 10^6$/mL, and for PGL patients $5.0 \pm 0.6 \times 10^6$/mL. The mean ± SD absolute neutrophil counts were $2.1 \pm 0.9 \times 10^6$/mL, $2.5 \pm 1.2 \times 10^6$/mL, and $3.0 \pm 0.6 \times 10^6$/mL for the patient groups, respectively. The WBC and neutrophil counts were not significantly different between the three HIV-infected groups. There was no significant difference between the WBC or neutrophil counts of the homosexual and drug abuser groups studied or between homosexual patients with *Pneumocystis carinii* pneumonia (PCP), Kaposi's sarcoma (KS), or other opportunistic infections (OIs). The WBC and neutrophil counts of AIDS and

Table 1. Leukocyte and Neutrophil Counts of Patients

Patients* (n)	Leukocyte Count (Range)	Neutrophils (Range)
AIDS (53)	3.3 ± 1.8 (1.0-8.6)	2.1 ± 0.9 (0.1-7.5)
ARC (20)	4.5 ± 1.7 (2.8-7.0)	2.5 ± 1.2 (1.2-4.8)
PGL (5)	5.0 ± 0.6 (4.2-5.9)	3.0 ± 0.6 (2.4-3.8)
AIDS, by risk group†		
Homosexual (38)	3.3 ± 1.6 (1.0-7.7)	2.1 ± 0.98 (0.13-4.2)
IVDA (13)	3.6 ± 3.4 (1.6-8.6)	3.4 ± 3.1 (1.2-7.5)
Hemophiliac (1)	1.5	0.7
Wife of IVDA (1)	5.0	4.0
Homosexuals with AIDS, by infection/neoplasm‡		
PCP (16)	2.9 ± 1.4 (1.1-5.2)	2.1 ± 1.3 (0.13-4.2)
Other OI (14)	3.5 ± 2.2 (1.0-7.7)	1.9 ± 0.8 (0.67-3.3)
KS (8)	3.9 ± 0.6 (1.1-5.2)	2.3 ± 0.4 (1.8-2.6)

Neutrophil counts are mean count × 10^6 ± 1 SD/mL. Normal leukocyte count = 7.8 (4.8-10.8) × 10^6/mL; normal neutrophil count = 4.0 (1.9-8.0) × 10^6/mL.

*Differences between AIDS and ARC counts were not significant.

†Differences between leukocyte and neutrophil counts of homosexuals and IVDA were not significant.

‡Differences between leukocyte and neutrophil counts of homosexuals with PCP, and other OIs, and KS were not significant.

ARC groups were significantly reduced when compared with those of the normal population ($P < .01$).

CFU-GM proliferation. As shown in Fig 1, BM cells from 53 AIDS patients, 20 ARC patients, and 5 PGL patients each generated significantly fewer CFU-GM colonies than did controls ($P < .01$). No significant differences were noted among AIDS, ARC, and PGL patients, among the various risk groups with AIDS described above, or when the patients with AIDS who had PCP, other OIs, or KS without OI were compared ($P > .5$ for each comparison), although each group was significantly reduced when compared with normal subjects ($P < .01$).

Coculture and FLs. Control PB-MNCs were cultured for CFU-GM in the presence of 10% serum from AIDS

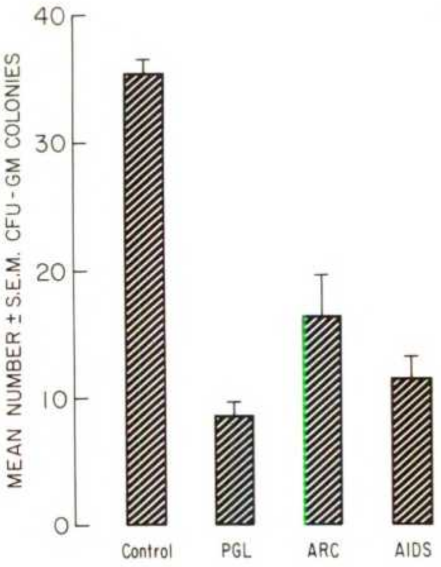

Fig 1. Comparison of growth of the granulocyte-macrophage progenitor cell colonies (CFU-GMs, mean ± SEM) from 9 healthy controls (35.7 ± 1.5), 5 patients with PGL (8.6 ± 1.1), 20 ARC (16.4 ± 3.2), and 53 AIDS (11.32 ± 1.7). There was a statistically significant difference ($P < .001$) in the number of CFU-GM colonies between the control group and each of the three groups of patients, but not between the patient groups. There was no significant difference between the patient groups. Within the AIDS group, there was no significant difference between the patients with an opportunistic infection (treated or untreated) and those with a neoplasm characteristic of AIDS (not shown). No patients had received antineoplastic chemotherapy prior to being studied.

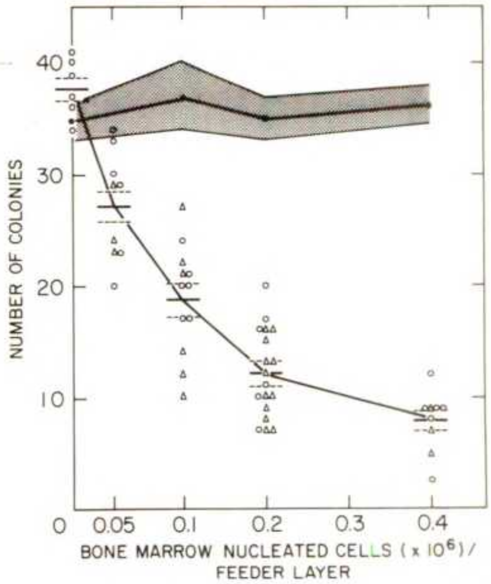

Fig 2. Dose-related inhibition of control CFU-GMs by BM-nucleated cells from AIDS patients. BM-nucleated cells from patients (n = 10) with AIDS were isolated in FLs at concentrations of 0.05, 0.1, 0.2, and 0.4 × 10^6 cells/mL and overlayered with 0.2 × 10^6 control BM-nucleated cells (○) or PB-MNCs (△). These control cells had progressive decreases in colony growth of 20.2%, 44.2%, 68.1%, and 80.1%, respectively, when compared with control cells grown over FLs devoid of cells (heavy lines are the mean number of colonies; broken lines are the SEM). Mean number of colonies grown over FLs containing normal control BM nucleated cells (●) (shaded area denotes SEM). The decrement seen for each group was significantly different ($P < .001$) from values for control cells grown over cell-free FLs or FLs containing 0.2 × 10^6 control BM cells.

patients. No difference was seen between these cultures and those cultured with or without heterologous control serum (data not shown). In contrast, coculture of AIDS BM-nucleated cells with control PB-MNCs in 0.3% agar revealed significant, sometimes total, colony growth inhibition ($P < .001$).

When FLs containing varying numbers of AIDS-BM nucleated cells were overlayered with 1×10^6 control PB-MNCs, CFU-GM growth at maximal inhibition was similar to that from the BM cells from the AIDS patients. Decreased colony formation by the control cells was seen in a dose–response fashion with increasing concentrations of patients' cells (Fig 2).

Conditioned media. Cell-free CM prepared from the BM-nucleated cells from eight patients with AIDS and two controls were added to the FLs at a final concentration of 10% in place of BM cells. The CM from all the AIDS patients significantly ($P < .001$) inhibited the CFU-GM growth of normal PB-MNCs in the same fashion as did AIDS BM-nucleated cells. Control CM had no effect on the cultures. After ammonium sulfate precipitation of the AIDS CM, the inhibitory activity was recovered from the precipitates (Fig 3) but not from the supernatants (not shown). Again, control CM showed no inhibitory activity in either fraction.

PAGE. The ammonium sulfate precipitates of the patients' and controls' CM were run in 7.5% polyacrylamide gels. A unique band was observed in the lanes containing the patients' CM but was absent in the control lanes (Fig 4). This protein, absent in the control lanes, had a relative molecular mass of 84 kd as determined by SDS-PAGE (Fig 4). PAS staining of these gels revealed a gp band in the same region as the unique band, but the 84-kd region was PAS negative in the control lanes.

The proteins were electrophoretically eluted from serial slices of 7.5% preparative PAGE. The proteins isolated in this manner had inhibitory activity in the CFU-GM assay in the same fashion as described for the CM. The inhibitory activity was recovered from proteins eluted from the region of the gel that contained the unique band (Fig 3). No other proteins with inhibitory activity were recovered from any other region of these lanes or from control lanes.

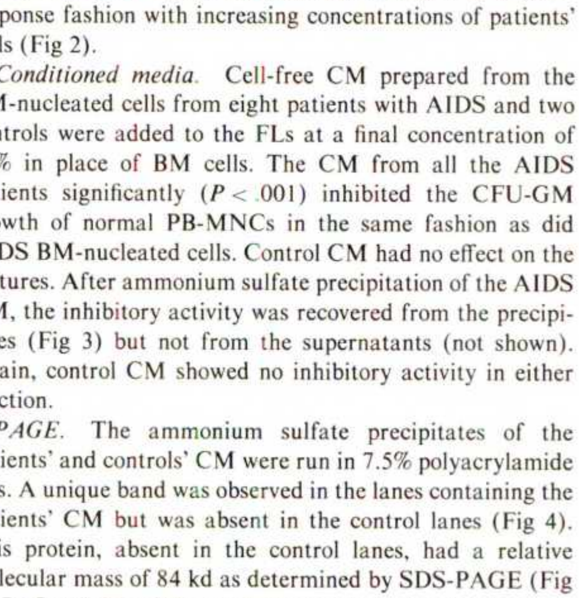

Fig 3. Control CFU-GM colony growth (mean ± SEM) over FLs that were without cells or CM (36.25 ± 1.1) or contained CM prepared from control BM cells (35.7 ± 1.5), 0.2×10^6 BM-nucleated cells from patients with AIDS (12.1 ± 2.0), CM prepared from AIDS BM cells (6.0 ± 0.6), resolubilized ammonium sulfate precipitate from AIDS BM CM (3.3 ± 0.7), and electrophoretic eluates of 84-kd bands from PAGE-separated CM (2.0 ± 1.2).

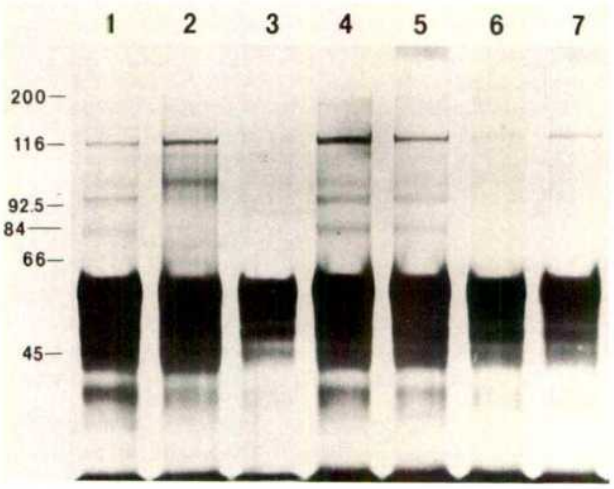

Fig 4. Resolubilized ammonium sulfate precipitates of CM were electrophoresed into a 7.5% SDS-polyacrylamide gel and silver stained to visualize the separated proteins. Lanes 1, 2, and 4 contained CM prepared from bone marrow (BM) cells of three different patients with AIDS. Lane 5 contained CM from a patient with ARC. Lane 7 contained CM from a healthy HIV-seronegative male homosexual. Lanes 3 and 6 contained CM from two different normal controls.

Western blotting. Resolubilized ammonium sulfate precipitates of CM were electrophoresed into 7.5% SDS-PAGE and were then blotted as described. The blotted proteins were then probed with polyclonal and monoclonal antibodies raised against HIV antigens (generously provided by Drs Robert Gallo and S. Zaki Salahuddin of the National Cancer Institute). The 84-kd band was not recognized by any of these antibodies.

DISCUSSION

Leukopenia and neutropenia are frequent findings in patients within the AIDS spectrum. To elucidate the mechanism of the neutropenia, we assessed the proliferation of CFU-GMs from the BM cells of 78 patients within the AIDS spectrum (AIDS, ARC, and PGL). BM cells from 70 of 78 patients made significantly fewer CFU-GM colonies than did controls ($P < .01$).

The number of CFU-GM colonies from groups of patients with AIDS, ARC, or PGL were not statistically significantly different from each other, indicating that this decrease was not related to the severity of clinical disease. Statistical comparison of the number of CFU-GMs among the various risk groups also showed no significant differences. Homosexual men, the largest group of AIDS patients we studied, were further divided into three subgroups: those with PCP, other OIs, or with KS with no OI. No significant differences were seen between these subgroups in the number of CFU-GM colonies, indicating that there was no relationship to any type of secondary infection or malignancy. Of particular interest, 60% of the patients with PCP but none of the others had been treated with trimethoprim-sulfamethoxazole. Although this drug combination has been well documented to cause neutropenia and AIDS patients have been reported to be especially sensitive,[6] these results from the various subgroups indicate that the neutropenia and reduced CFU-GMs in our patients were not consequent to such drug therapy. The apparent exquisite sensitivity to the drug may be the result of a background of preexisting defective granulopoiesis with little or no reserve capacity when even minor additional suppression is superimposed.

Coculture of control PB-MNCs with AIDS BM cells revealed a profound decrease in the number of colonies formed. To determine whether this decrease in CFU-GM colony growth was due to a cell–cell interaction or to a factor released by the cells into the culture milieu, patients' BM-nucleated cells were immobilized in a FL without CSF, over which the control cells were cultured with added CSF. Because the normal CFU-GMs can be progressively inhibited with increasing concentrations of physically separated patients' cells in the FL, we established the humoral nature of the suppression.

To identify further the factor(s) responsible, CM were obtained from liquid cultures of AIDS BM-nucleated cells. CM added to cell-free FLs inhibited growth to a degree comparable to coculture with BM cells (Fig 3). The inhibitory factor was precipitated by 50% ammonium sulfate. PAGE analysis of the CM precipitate and PAS staining revealed that the inhibitory factor is a gp with a relative mol wt of 84 kd. The 84-kd bands electrophoretically eluted from the gels had inhibitory activity identical to that of the CM.

We previously reported that excessive margination of neutrophils was not found in AIDS pateints and that five of six AIDS patients had a decreased mobilizable BM neutrophil storage pool in vivo.[23] Morphological observations of BM biopsies confirmed a decrease in the mature neutrophil pool.[24] Because the fraction of BM myeloid precursors in DNA synthesis was within the normal range, BM granulopoiesis may not respond appropriately to the neutropenia. Although our data from in vitro cultures cannot be directly compared with the above observations in vivo, the decrease in the BM storage pool of mature neutrophils could be explained at least in part by inhibition of stem cell activity. The inhibitory activity on BM neutrophil progenitors demonstrated in vitro might also affect more mature precursors. The decrease in the storage pool, in turn, would explain a profound decrease in CFU-GMs associated with a less profound decrease in blood neutrophils since one can maintain blood neutrophils at the expense of the storage pool.

Donahue et al[25] have reported that sera and immunoglobulins from HIV-infected individuals suppressed CFU-GMs and BFU-Es from AIDS and ARC patients but not from healthy controls. They observed that antibodies against the HIV envelop gp130, inhibit CFU-GM, thus suggesting that this antibody reacts with HIV infected progenitors and suppresses them. Similarly, we found that sera from patients with AIDS had no effect on CFU-GMs from healthy controls, but the novel gp that we report here differs from the findings of Donahue et al since it is not an immunoglobulin and is not cross-reactive with antibodies against HIV. In other unpublished studies, we found no evidence of inhibition of BM cells or PB BFU-Es or CFU-Es by control or patient CM. In addition, CM added to control PB-MNCs stimulated with varying concentrations of phytohemagglutin, pokeweed mitogen, and concanavalin-A did not decrease the blastogenic response to these mitogens. Thus, the specificity of gp84 is different from that reported by Donahue et al and from an inhibitor of lymphocyte proliferation in AIDS patients described by Laurence and Mayer.[26] Our Western blot data do not preclude the possibility that gp84 is an HIV product, although it is more likely a secondary product of HIV infection.

Inhibitors of CFU-GMs have been well described in numerous acute diseases and syndromes.[27-30] Many of these inhibitors are T lymphocyte-mediated,[31-34] whereas others are factors released by other specific cell populations.[35-41] The gp that we demonstrated has different physiochemical characteristics from these previously described factors.

FeLV, a retrovirus with similarities to HIV, induces an immune-deficient state in some infected cats, many of whom are neutropenic as well.[42,43] These infected, neutropenic animals have a decrease in the number of BM CFU-GMs, although no explanatory mechanism has been proposed.[44] Conceivably, the neutropenia secondary to FeLV infection is analogous to the neutropenia associated with AIDS.

Our demonstration of a gp that inhibits granulopoiesis in vitro possibly provides an explanatory mechanism for previous observations of neutropenia in patients within the AIDS spectrum.

Preliminary experiments suggesting the specificity of the inhibitor raise the possibility that a series of specific viral products or virally induced products may explain some of the many cellular defects observed in these patients. The lack of reactivity with known antibodies to HIV suggests that gp84 is not a viral constituent, although gp84 may be produced by a population of BM cells in response to HIV infection.

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Molecular Genetic Survey of 16 Kindreds With Hereditary Antithrombin III Deficiency

By Susan Clark Bock and Edward V. Prochownik

Molecular genetic techniques were utilized to examine antithrombin III (ATIII) gene status in 16 independently ascertained kindreds with hereditary ATIII deficiency. In one of these families antithrombin III deficiency is caused by hemizygosity of the ATIII locus. In the remaining 15 kindreds, two copies of the ATIII gene are present and appear to be grossly normal at the level of whole genome Southern blotting, suggesting that small deletions, insertions or limited nucleotide substitution(s) in the antithrombin III gene, or "trans-acting" defects at other loci involved in the processing, modification, and secretion of biologically active ATIII are responsible for the observed anticoagulant disorders.

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ANTITHROMBIN III (ATIII) is a plasma glycoprotein that is in large part responsible for maintaining the fluidity of blood. It inhibits thrombin and other activated proteinases of the coagulation cascade by forming stoichiometric 1:1 inhibitor–enzyme complexes with them.[1] The rate of antithrombin III-mediated thrombin inactivation is modulated by the acidic mucopolysaccharide heparin, increasing by over three orders of magnitude in the presence of this cofactor.[1]

Studies of families with inherited antithrombin III deficiency have provided evidence that ATIII is an important physiologic inhibitor of thrombin.[2] Affected individuals in such families have functional anticoagulant levels that are about 50% of normal and tend to suffer recurring episodes of pulmonary embolism and deep vein thrombosis during adult life. More than 50 families with ATIII deficiency have been reported in the literature (see Thaler and Lechner[3] for review). The human ATIII structural gene locus maps to chromosome 1, subregion 1q23-q25,[4] and the mode of inheritance in all cases of ATIII deficiency studied so far is autosomal dominant.

Two categories of ATIII deficiency have been defined on the basis of ATIII antigen levels in the plasma of affected individuals. The majority of ATIII deficiency kindreds belong in the type I ("classical") deficiency group[5] and have a quantitatively abnormal phenotype in which antigen and heparin cofactor levels are both reduced to about 50% of normal. Possible molecular genetic explanations for type I deficiencies include complete or partial deletion or rearrangement of one ATIII allele or mutations that interrupt anticoagulant biosynthesis at the RNA or protein level.

The second category of ATIII deficiency affects about 20% of families with hereditary thrombosis[6] and has been termed type II ("functional") deficiency.[5] Affected individuals from these kindreds produce dysfunctional ATIII molecules; they have reduced heparin cofactor activity levels (approximately 50% of normal) but levels of ATIII antigen are often normal or nearly normal.[7-9] In addition, variant ATIII molecules, but not thrombotic symptoms, are inherited in several other families.[20-23] Studies of dysfunctional and variant ATIIIs have shown that the abnormalities in ATIII molecules from different kindreds are heterogeneous, and the amino acid substitutions responsible for dysfunction have been identified in four cases.[16,24-26]

cDNA clones for human ATIII have been isolated,[27-29] and the structure of the gene has been determined.[30] In addition, two restriction fragment length polymorphisms in the ATIII locus have been identified.[31,32] The availability of probes, a map, and linkage markers for the ATIII gene have made it feasible to use a molecular genetic approach to study the origins of ATIII deficiency in affected families. This communication reports the initial results of a survey on 16 different kindreds that segregate hereditary ATIII deficiency.

METHODS

ATIII deficient families. Family histories, hematologic data, and blood samples were kindly supplied by Drs K. Bauer (Boston), J. Brandt (Columbus, OH), J. Chediak (Chicago), J. Lewis (Pittsburgh), J. Rand (New York), M. Skolnick (Salt Lake City), J. Weitz (New York), and G. White and J. Griffith (Chapel Hill, NC). Reports on kindreds RU54,[10] RU 90,[19,24,33] CH1 and CH2,[31] and CH6[34] have appeared previously in the literature.

Preparation of genomic DNA and Southern blots. High mol wt genomic DNA samples were prepared as previously described[19,30] from 30-cc blood samples anticoagulated with EDTA or acid-citrate-dextrose. Five-to 15-microgram DNA samples were digested with the indicated restriction enzymes and used to prepare Southern blots[35] on nitrocellulose or nylon filters following electrophoresis through 0.8% or 1.0% agarose gels. The Southern blots were hybridized to antithrombin III cDNA probes[27,28] that had been labeled to high specific activity by nick translation with ^{32}P.

Gene dosage studies. ATIII gene dosage was determined in several different ways. In some individuals the presence of two ATIII alleles could be visualized directly on Southern blots using alleles of the ATIII sequence[31] and length[32] DNA polymorphisms as linkage markers. Similarly, a family study of DNA polymorphism segregation was used to demonstrate the deletion of one ATIII allele in one kindred.[31] Finally, quantitative ATIII gene dosage studies were performed on those families in which all available affected individuals were not heterozygous for either of the restriction fragment length polymorphisms (ie, they were either homozygous or

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hemizygous at these markers). In these studies Southern blots were hybridized to a mixed probe containing human ATIII[27] and C$\bar{1}$ inhibitor[36] cDNA sequences. ATIII is located in subregion q23-q25 of chromosome 1,[4] while C$\bar{1}$ inhibitor maps to chromosome 11, q12-q13.1.[45] C$\bar{1}$ inhibitor thus serves as an internal gene dosage and blotting control in this protocol. Control DNA for the quantitative gene dosage experiment was extracted from cultured fibroblasts of the GM2025 cell line (del 1q21-1q25; Human Genetic Mutant Repository, Camden, NJ). Following autoradiography films were scanned on a densitometer.

RESULTS

The flow chart shown in Fig 1 depicts the experimental scheme used to study ATIII gene status in families with hereditary ATIII deficiency. Table 1 summarizes the results of our work on 16 such families.

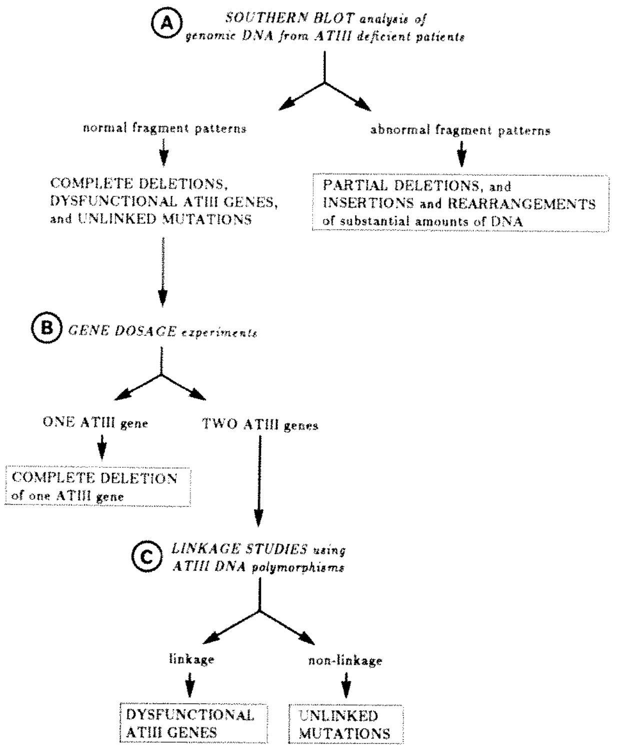
A
SOUTHERN BLOT analysis of genomic DNA from ATIII deficient patients
normal fragment patterns
abnormal fragment patterns
COMPLETE DELETIONS, DYSFUNCTIONAL ATIII GENES, and UNLINKED MUTATIONS
PARTIAL DELETIONS, and INSERTIONS and REARRANGEMENTS of substantial amounts of DNA
B
GENE DOSAGE experiments
ONE ATIII gene
TWO ATIII genes
COMPLETE DELETION of one ATIII gene
C
LINKAGE STUDIES using ATIII DNA polymorphisms
linkage
non-linkage
DYSFUNCTIONAL ATIII GENES
UNLINKED MUTATIONS

Fig 1. Flow chart showing experimental procedures used for molecular genetic analysis of hereditary antithrombin III deficiencies and their possible outcomes.

Table 1. AT III Levels and AT III Gene Status in 16 Families With Hereditary Antithrombin III Deficiency

Kindred	ATIII Functional Activity	ATIII Antigen Level	ATIII Gene Dosage	Type of Gene Defect	Polymorphism Haplotype if Dysfunctional
RU 51	R	R	2*	DG or UL	−,F
RU 52/56	R	R	2†	DG or UL	−,F
RU 53	R	R	2*	DG or UL	−,F
RU 54	R	N	2*	DG or UL	−,F
RU 55	R	ND	2*	DG or UL	−,F
RU 57	R	R	2*	DG or UL	−,F
RU 58	R	N	2*	DG or UL	−,F
RU 59	R	ND	2*	DG or UL	+,F
RU 60	R	ND	2*	DG or UL	−,F
RU 90	R	R	2†	DG	+,S
CH1	R	R	1*‡	CD	
CH2	R	R	2†	DG or UL	+,S
CH3	R	R	2*	DG or UL	+,F
CH4	R	R	2†	DG or UL	+,S
CH5	R	R	2†	DG or UL	+,F
CH6	R	R	2†	DG or UL	+,F

Abbreviations: R, reduced; N, normal; ND, not determined; DG, dysfunctional gene; UL, unlinked, *trans*acting mutation; CD, complete deletion.

*Method of gene dosage determination: Quantitative dosage blot.

†Method of gene dosage determination: DNA polymorphism heterozygosity in affected individual.

‡Method of gene dosage determination: Family study using DNA polymorphism.

Absence of gross abnormalities in the ATIII gene. Southern blot analysis (experiment A on flowchart, Fig 1) can resolve gross alterations in a locus of interest, since the deletion, insertion, or rearrangement of a substantial amount of DNA ($>\sim$100 bp) will alter the distance between adjacent restriction sites and generate new hybridizing fragments with unusual mobilities. A battery of restriction enzymes (BamHI, EcoRI, HindIII and PstI) known to cleave collectively at fairly regular intervals throughout the ATIII locus[30] was used to digest normal and patient DNAs. Southern blots were prepared and hybridized to an ATIII cDNA probe. In the blot shown in Fig 2 and others, the patterns of hybridizing fragments obtained from DNAs of affected individuals in all 16 deficiency kindreds were qualitatively identical to those produced by DNAs from normal relatives and controls after variations caused by commonly occurring DNA polymorphisms had been accounted for. These data indicate that ATIII deficiency in these families is not caused by insertion or rearrangement of a substantial amount of DNA in the ATIII locus or partial deletion of the gene but suggest (1) complete deletions, (2) point mutations, or (3) *trans*-acting, unlinked mutations as possible causes of hereditary thrombosis.

Antithrombin II gene dosage in ATIII deficient families. The next stage in our analysis of ATIII deficiency was to determine ATIII gene dosage in the 16 families with hereditary thrombosis (experiment B on flowchart, Fig 1). The issue of whether hemizygosity of the ATIII locus was responsible for reduced antithrombin levels was assessed (1) by using ATIII DNA polymorphisms as linkage markers for specific antithrombin III alleles and (2) through quantitative Southern blot experiments.

Two two-allele DNA polymorphisms[31,32] have been identified with ATIII cDNA hybridization probes. The "+" and "−" alleles of the PstI sequence polymorphism generate a hybridizing 5-kb doublet and a 10-kb band, respectively (Fig 3 A), while the "F" and "S" alleles of the length polymorphism produce 1450 and 1550 nucleotide BamHI fragments, respectively (Fig 3 B). Figure 3 C shows blots prepared with PstI- (top) and BamHI-cleaved DNA (bottom) from two affected members of the RU52/56 kindred. The DNA sample from patient 1 produced a 10-kb PstI band and a 1450 nucleotide BamHI fragment. This pattern is consistent with the presence of one or two −,F haplotype alleles. However, polymorphism analysis of DNA from another

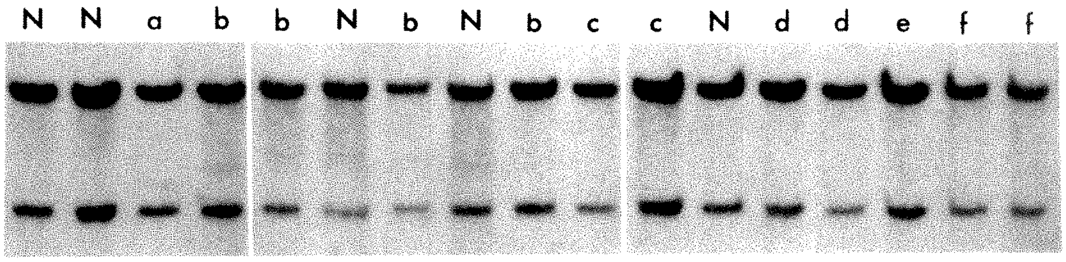

Fig 2. Southern blot of DNAs from normals and ATIII-deficient patients. The blot was prepared with EcoRI-digested genomic DNAs and hybridized to ^{32}P-labeled ATIII cDNA insert sequences. Each lane shown contains DNA from a different individual. N, normals; *a*, affected patient from RU51; b, four affected patients from RU52/56; c, two affected patients from RU53; d, two affected patients from RU54; e, affected patient from RU55; f, two affected patients from RU90.

affected member of the family, patient 2, showed that deletion of one ATIII gene has not occurred in this kindred. The presence of two ATIII genes can be directly visualized in individual 2 as heterozygosity at both the sequence and length polymorphisms. Identification of affected individuals who are heterozygous at one or both ATIII DNA polymorphisms was also used to exclude hemizygosity as the cause of antithrombin III deficiency in CH2,[31] CH4, CH5, CH6, and RU90.[19] Similarly, inheritance of DNA polymorphisms was used to demonstrate hemizygosity of the ATIII locus in another family. The presence of a null allele was deduced by monitoring the segregation of the PstI sequence polymorphism in CH1 and established that the hereditary tendency to thrombosis in this kindred is due to complete deletion of one ATIII allele.[31]

Quantitative blotting experiments were used to determine ATIII gene dosage in the families in which analysis of ATIII DNA polymorphisms was not productive due to the lack of marker heterozygosity in available affected patients. Southern blots prepared from HindIII-digested DNAs were hybridized to a mixed probe containing ATIII cDNA and C$\bar{1}$ inhibitor cDNA sequences. The latter probe, which maps to chromosome 11,[36] is unlinked to ATIII (which maps to chromosome 1[4]) and served as a DNA dosage and transfer control in this experiment. Autoradiograms of the blots were scanned on a densitometer and normalized so that the intensities of the 4-kb bands resulting from control probe hybridization equaled 100%. Some traces are shown in Fig 4. When samples from affected individuals in the RU51, RU53, RU54, RU55, RU57, RU58, RU59, RU60, and CH3 pedigrees were analyzed, intensities of the 2.2-kb and 2.5-kb ATIII fragments were equal to those of the corresponding bands in samples from unaffected, normal controls and double those observed in a sample from the ATIII deletion control GM2025 (del 1q21-1q25). Therefore, two ATIII genes are present in patients from these nine families, and hemizygosity of the antithrombin III locus is not the cause of anticoagulant deficiency. Analysis of a sample from CH1, however, reconfirmed that affected patients in this family have only one ATIII gene.[31]

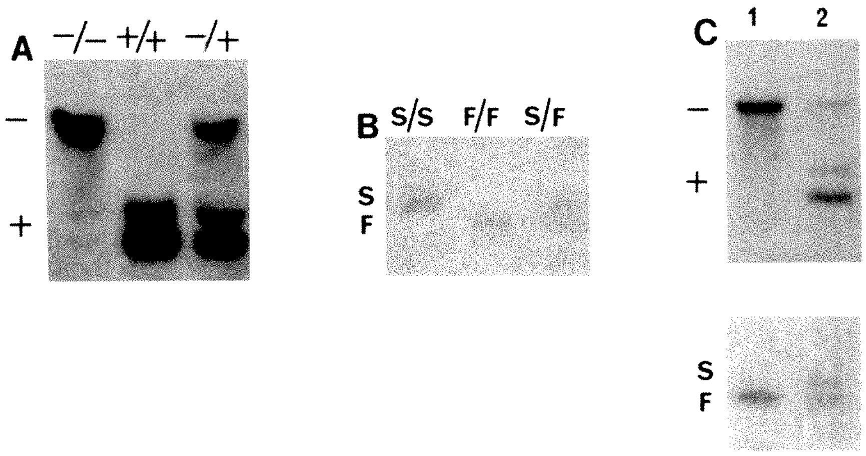

Fig 3. Use of ATIII DNA polymorphisms to determine antithrombin III gene dosage. Southern blots were prepared from PstI- (a and c, top panel) and BamHI- (b and c, bottom panel) digested DNAs and hydridized to ^{32}P-labeled ATIII cDNA sequences. (a) Sequence polymorphism. "−/−" homozygotes produce a 10-kb band (left), while "+/+" homozygotes generate a 5-kb doublet (center), and "−/+" heterozygotes produce a composite pattern (right) in which bands are approximately one half as intense as in homozygotes. (b) Length polymorphism. "S/S" homozygotes produce a 1550-bp band (left), "F/F" homozygotes a 1450-bp band (center), and "S/F" heterozygotes show both fragments at reduced intensity (right). (c) Affected individuals in kindred RU52/56 have two copies of the ATIII gene. Sequence polymorphism data are shown in the top panel and length polymorphism data in the bottom panel. The haplotype of patient 1 could be "−,F/−,F" or "−,F/null". However, the haplotype of patient 2 is "−,F/+,S", indicating that deletion of one ATIII allele has not occurred.

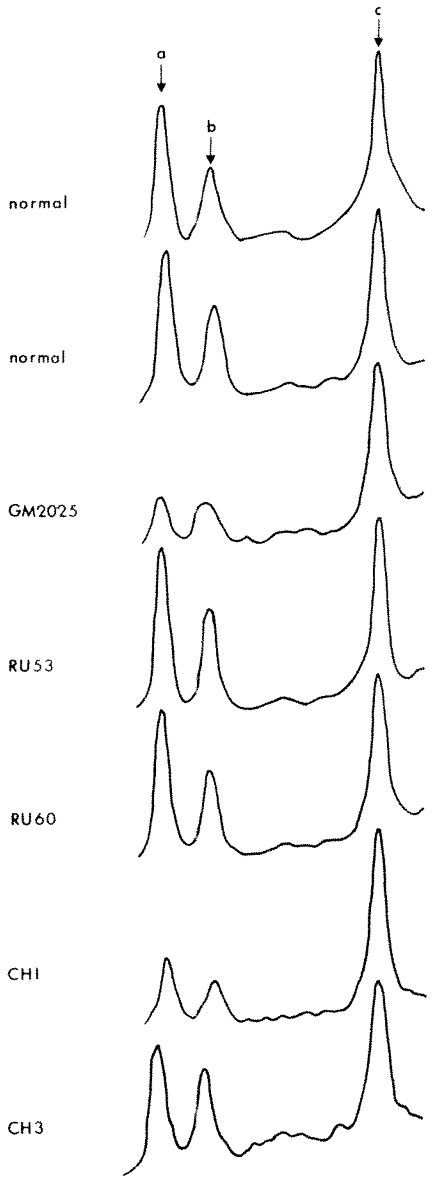

Fig 4. Quantitative ATIII gene dosage experiment. Southern blots were prepared from the indicated HindIII-digested genomic DNAs and hybridized to a mixed probe containing ATIII and C$\overline{1}$ inhibitor cDNA sequences. Following autoradiography the film was scanned on a densitometer. Peaks a and b are 2.2- and 2.5-kb bands resulting from hybridization to the ATIII cDNA probe. Peak c is a 4-kb band resulting from hybridization to the C$\overline{1}$ inhibitor control probe, and its height has been normalized to 100% in the tracings shown. Intensities of peaks a and b from patient samples of RU53, RU60, and CH3 correspond to those observed in normals and indicate that two copies of the antithrombin III gene are present. Complete deletion of one ATIII allele in the deletion control sample (GM2025, del 1q21-q25) and in ATIII deficiency kindred CH1 is indicated by reduced intensity of peaks a and b in these scans.

DISCUSSION

Table 1 summarizes the results of our series of 16 ATIII deficiency families. Complete deletion of the ATIII locus has occurred in one case (CH1). Affected members of this family have reduced functional activity and immunologic levels of ATIII, and one individual has experienced deep vein thrombosis. Half normal levels of antithrombin III have also been observed in patients with large, cytologically visible deletions of 1q (del 1q22-q25 and del 1q21-q25).[37]

Major structural gene alterations (partial deletions, rearrangements, and duplications) have been established as the molecular causes of genetic disease in certain patients with thalassemia,[38] Lesch-Nyhan syndrome,[39] apolipoprotein AI deficiency,[40] and hemophilia.[41] However, among the families we studied there were no examples of large gene alterations in the ATIII locus causing anticoagulant deficiency. The hybridization patterns of normal and patient DNAs were determined using four different restriction enzymes that are known to cleave collectively at fairly regular intervals throughout the ATIII gene, and we believe that changes affecting ≥100 bp of DNA would have been detected.

Affected individuals in 15 of the 16 families surveyed here had two ATIII genes. In these cases ATIII deficiency may be caused by a small mutation in the ATIII gene itself or by a defect in a transacting factor involved in the synthesis, maturation, or secretion of the functional anticoagulant molecule. These two alternatives can be distinguished by linkage tests (experiment C on flowchart in Fig 1). In the case of dysfunctional ATIII genes, ATIII deficiency will cosegregate with a given ATIII allele (which can be recognized by its DNA polymorphism haplotype). The disorder and specific ATIII alleles will show unlinked inheritance in the cases in which anticoagulant deficiency is caused by the dysfunction of a transacting factor or the gene encoding it. Since only two two-allele DNA polymorphisms have been identified in the ATIII locus (combined polymorphism information content = 0.45[19]), extensive family studies are required to confidently distinguish the dysfunctional gene and unlinked mutation alternatives and have been completed in only one case so far (RU 90).

RU 90 is a large Utah kindred in which 13 members in 3 generations have had ATIII deficiency. This family was originally described as a type I deficiency, since ATIII antigen and activity levels are both reduced to approximately 50% of normal.[33] However, subsequent immunoblotting experiments revealed the presence of very small amounts of a variant ATIII molecule in the plasma of Utah patients, and a maximum likelihood linkage study established that a dysfunctional gene residing on a chromosome of the +,S ATIII DNA polymorphism haplotype encodes this electrophoretically abnormal inhibitor.[19] The ATIII-Utah allele has been isolated by molecular cloning, and a C to T mutation has been identified in the second position of the codon for proline-407,[24,42] a highly conserved residue in the serine protease inhibitor gene family.[43] The resultant leucine for proline substitution may cause rapid turnover of the mutant gene product (due to incorrect folding) and/or loss of the ability to form functional inhibitor-protease complexes with thrombin (since proline-407 is located only 14 residues away from the reactive site arginine[44]).

Molecular genetic techniques have been used to survey ATIII gene status in a series of 16 kindreds with ATIII deficiency. Knowledge of the genetic heterogeneity and mutations underlying ATIII deficiencies will allow improvements in the diagnosis and treatment of affected individuals. Additionally, characterization of functionally abnormal ATIII molecules and the genes encoding them will contribute to our understanding of how this anticoagulant molecule works and how it is activated by heparin.

ACKNOWLEDGMENT

We thank members of the ATIII-deficient families and their physicians for participating in this study and Dr Stuart Orkin for support and helpful advice. The expert technical assistance of J.F.Harris, D.J. Levitan, J.A. Marrinan, and E. Radziejewska is gratefully acknowledged.

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Oncoplacental Protein SP$_1$—A Constitutive and Inducible Late Differentiation Marker of the Human Myelomonocytic Lineage

By Markku Heikinheimo, Carl G. Gahmberg, Hans Bohn, and Leif C. Andersson

The oncoplacental protein SP$_1$ is found in large quantities in human placenta, amniotic fluid, and pregnancy serum. Low levels have been reported in association with malignancy but also in healthy nonpregnant individuals. By indirect immunofluorescence, fluorescence-activated cell sorting, and immunoprecipitation we here demonstrate the presence of SP$_1$ both on the surface and in the cytoplasm of human granulocytes but not in earlier myeloid progenitor cells in bone marrow. Lymphocytes did not contain the protein, and only trace amounts could be found in the cytoplasm of blood monocytes. A major glycoprotein with an apparent mol wt of 90,000 was obtained by immunoprecipitation of surface-labeled granulocytes. Cultivated blood monocytes, while adhering to surfaces or forming multinuclear giant cells, displayed a strong membrane and cytoplasmic expression of SP$_1$. Treatment of the myeloid leukemia cell line ML-2 with tetraphorbol acetate (TPA) strongly induced SP$_1$ in the membrane and cytoplasm as revealed by immunofluorescence and polyacrylamide gel electrophoresis (PAGE) of immunoprecipitates from lysates of surface radiolabeled cells. The induction of synthesis of SP$_1$ in TPA-treated cells was confirmed by immunoprecipitation from lysates of cells metabolically labeled with ^{35}S-methionine. Human lymphoblastoid and erythroleukemic cell lines did not express SP$_1$ either before or after induced differentiation. Thus SP$_1$ provides a late differentiation marker for the myelomonocytic lineage and is strongly induced during macrophage differentiation or by TPA treatment of ML-2 cells.

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THE PREGNANCY-SPECIFIC β-$_1$-glycoprotein SP$_1$ is a glycoprotein with an apparent mol wt of 90,000, and it was originally isolated from human placenta.[1] While human serum normally contains very low amounts of the protein,[2] serum from women in the last trimester of pregnancy contains up to 300 mg/L.

Immunohistochemical investigations have revealed the presence of SP$_1$ in normal and malignant trophoblasts. SP$_1$ has also been detected immunohistochemically in tumor tissue of nontrophoblastic origin, such as in neoplasms of the breast and gastro-intestinal (GI) tract.[3] Syncytiotrophoblasts have been shown to synthesize SP$_1$ in organ and cell cultures.[4,5] SP$_1$ has also been reported to occur in cultured fibroblasts and amniotic epithelial cells.[6,7]

Inaba et al[8] used histocytochemical techniques and found that an antiserum against SP$_1$ reacted with human and monkey blood neutrophils. The antigen(s) responsible for this immunoreactivity was not further characterized.

We have now investigated the occurrence and the regulation of expression of SP$_1$ in human hematopoietic cells and in leukemia cell lines before and after induction of differentiation.

MATERIALS AND METHODS

Hematopoietic cells. Normal blood leukocytes were isolated from buffy coats of blood units kindly donated by the Finnish Red Cross Blood Transfusion Service. Normal bone marrow was recovered from pieces of ribs that had to be resected during open thorax surgery.

Fractionation of cells. Blood granulocytes were isolated by dextran sedimentation of buffy coats. The leukocyte-rich suspension was subjected to Ficoll-Isopaque density gradient centrifugation at 400 g for 30 minutes. The lymphocytes and monocytes were recovered from the interphase, while the granulocytes were recovered from the pellet as described.[9] Monocytes were purified by Percoll density gradient centrifugation[10] of blood mononuclear cells recovered by Ficoll-Isopaque gradient centrifugation.

Cell lines. The following human hematopoetic cell lines were studied: the erythroleukemic lines K562[11,12] and HEL[13], the myeloid cell lines ML-2[14] and HL-60[15], and lymphoblastoid cell lines established locally by Epstein-Barr virus (EBV) infection of normal blood B cells.

Cell culture and induction of cell differentiation. Monocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and allowed to attach on glass coverslips that were used for immunofluorescent staining. Primary monolayer cultures of monocytes were detached with a rubber policeman and subcultured at high density for five days to form multinuclear giant cells on coverslips. HL-60 and ML-2 cells were cultivated at optimal densities for three to five days in the presence of 10^{-8} mol/L tetraphorbol acetate (TPA) or 10^{-7} mmol retinoic acid as described.[16] K562 and HEL cells were treated with 1.2 µg/mL sodium butyrate or hemin at 50 µg/mL as described.[17,18]

Immunofluorescence. Cytocentrifuged smears were fixed for five minutes in cold methanol (−20°C) to permeabilize the plasma membrane. The indirect immunofluorescence staining was done with 1 : 30 diluted rabbit anti-SP$_1$ (Dakopatts, Copenhagen, Denmark) followed by 1 : 20 diluted fluorescein isothiocyanate (FITC)-conjugated goat antirabbit antiserum. Double immunofluorescence staining was performed with the monoclonal antibody (MoAb) OKM-1[19] (Ortho, Raritan, NJ) and rabbit anti-SP$_1$ antiserum by using FITC-conjugated goat antimouse and rhodamine-conjugated goat antirabbit antisera, respectively.

Fluorescence-activated cell sorting. Normal bone marrow cells in suspension were stained in indirect immunofluorescence by rabbit anti-SP$_1$ antiserum. The cell suspension was fractionated on a FACS IV (Becton-Dickinson) cell sorter, and the positively stained and

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unstained populations were identified from May-Grünwald–Giemsa-stained cytocentrifuged smears.

Cell surface labeling, metabolic labeling, and immunoprecipitation. Purified granulocytes and uninduced and induced ML-2 cells (50×10^6 cells) were radioactively surface-labeled by the periodate/NaB^3H_4 technique, as described previously.[20] After labeling, the cells were solubilized at 0°C in 1 mL of 0.15 mol/L NaCl-0.01 mol/L sodium phosphate, pH 7.4, containing 1 % Triton X-100. The samples were centrifuged at 10,000 g for 15 minutes and the supernatants recovered. Twenty-five microliters were used for slab gel electrophoresis and 100-μL aliquots for immunoprecipitation. For metabolic labeling of ML-2 cells the cultured cells were incubated in 5 ml methionine-free Eagle's minimum essential medium (MEM) containing 1 mCi ^{35}S-methionine for one hour. After a two-hour chase in normal medium the cells were collected, washed twice, and lysates prepared for immunoprecipitation. Part of the ^{35}S-methionine-labeled samples were subjected to lentil lectin-Sepharose chromatography and the α-methylmannoside-eluted fractions used for immunoprecipitation.

Immunoprecipitations with protein A-containing staphylococci were performed using 10 μL rabbit anti-SP_1 antiserum in the absence or presence of 10 μg SP_1 protein. The procedure has been described in detail previously.[21]

Polyacrylamide gel electrophoresis ^{3}H-labeled cells and the immunoprecipitates were electrophoresed on 8% polyacrylamide slab gels,[22] and the radioactive proteins were visualized by fluorography as described.[23]

RESULTS

When cytocentrifuge smears from normal blood leukocytes were stained by immunofluorescence with rabbit anti-SP_1 antiserum, a strong intracytoplasmic staining was seen in granulocytes. No staining was obtained with lymphocytes. The eosinophilic granulocytes also remained unstained. In bone marrow a strong positive reactivity was obtained only with cells of the granulocytic lineage. The earliest myeloid cells staining with anti-SP_1 antiserum were found to be promyelocytes. Metamyelocytes and mature neutrophils showed a strong reactivity. No staining was seen in the erythroid or megakaryocytic lineages (Fig 1 A).

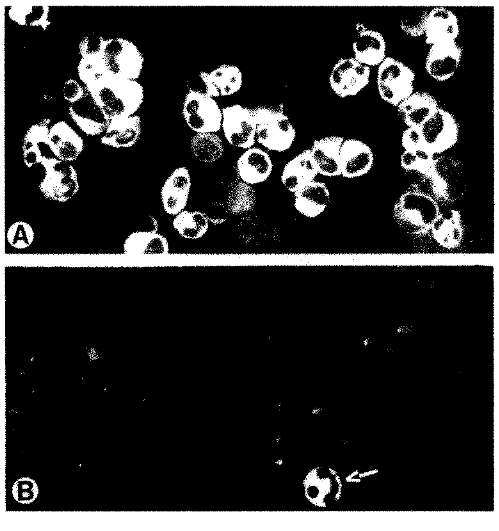

Fig 1. Immunofluorescence staining for SP_1 (rabbit anti-SP_1 antiserum) in normal bone marrow cells (A) and purified blood monocytes with one contaminating neutrophilic granulocyte (arrow, B).

Purified blood monocytes were negative in immunofluorescent surface staining, while a very faint, cytoplasmic reactivity, probably mainly in the Golgi area, could be seen in smears of monocytes (Fig 1 B).

Double-immunofluorescence surface staining with OKM-1 antibody and rabbit anti-SP_1 antiserum revealed a codistribution of these antigens as regards the granulocytic lineage, except that eosinophilic granulocytes were OKM-1 positive but SP_1 negative (figure not shown). Neither reagent detected early myeloblasts or cells of the erythroid lineage. Lymphoid cells were negative.

Sorting of anti-SP_1-stained bone marrow cells by a FACS IV revealed two populations (Fig 2 A). The positively staining cells consisted of mature granulocytes concentrating in the right shoulder of the histogram and their late precursors (Fig 2 C), while other bone marrow cells sorted in the unstained population (Fig 2 B).

When monocytes were allowed to adhere to a glass surface and were stained with anti-SP_1 antiserum, a strong intracytoplasmic reactivity was seen after only one day in culture (Fig 3 A). The staining intensity increased during the subsequent days of culture. Adherent monocytes forming multinuclear giant cells also displayed an intense and often asymmetrically distributed cytoplasmic and surface reactivity with the anti-SP_1 antiserum (Fig 3 C).

In the hematopoietic cell lines studied, SP_1 positivity was confined to the myeloid cell lines (ML-2 and HL-60). A very faint, hardly detectable, intracytoplasmic fluorescence like that in monocytes was seen in uninduced ML-2 (Fig 3 E) and HL-60 (not shown) cells. After treatment with TPA a very

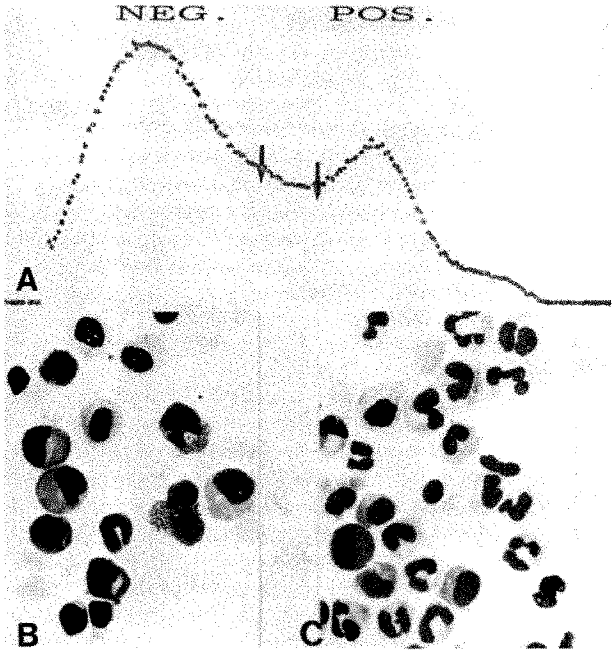

Fig 2. Fluorescence-activated cell sorting profile of normal human bone marrow utilizing rabbit anti-SP_1 antiserum (A). The SP_1 negative and positive cell populations are shown in B and C, respectively.

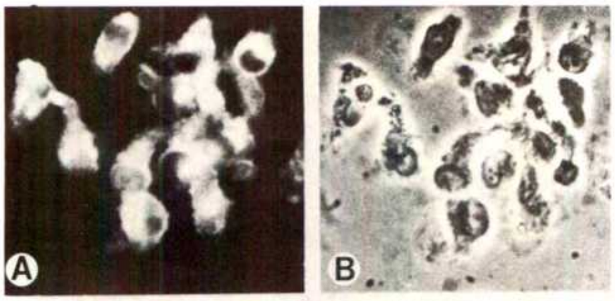

Fig 3. Immunofluorescence staining for SP$_1$ in adherent human monocytes (A), giant cells formed by monocytes (C), and the corresponding phase contrast microscopic presentations (B,D). The bottom figures show immunofluorescence staining for SP$_1$ in the myeloid cell line ML-2 before (E) and after (F) induction of cell differentiation by means of TPA.

strong membrane and cytoplasmic reactivity with anti-SP$_1$ was seen in ML-2 cells (Fig 3 F). TPA-treated HL-60 cells also showed an increased reactivity, while the lymphoblastoid and erythroleukemic cell lines did not stain with anti-SP$_1$ antiserum either before or after induced differentiation (not shown).

When immunoprecipitation was performed with anti-SP$_1$ antiserum from ^{3}H surface-labeled granulocytes and the precipitate electrophorized on polyacrylamide slab gels, two bands were seen. Most of the precipitated material displayed an electrophoretic mobility corresponding to an apparent mol wt of 90,000. In addition, a weaker band with an apparent mol wt of 54,000 was visualized (Fig 4 C). This might represent a proteolytic fragment. The addition of competing purified placental SP$_1$ protein to the lysate before the immunoprecipitation completely inhibited the specific reaction (Fig 4 D). Immunoprecipitation with anti-SP$_1$ antiserum from lysates of surface labeled, TPA-treated ML-2 cells gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 90,000 mW (Fig 5 G). No band was visualized from untreated or retinoic acid-treated ML-2 cells (Fig 5 E,F).

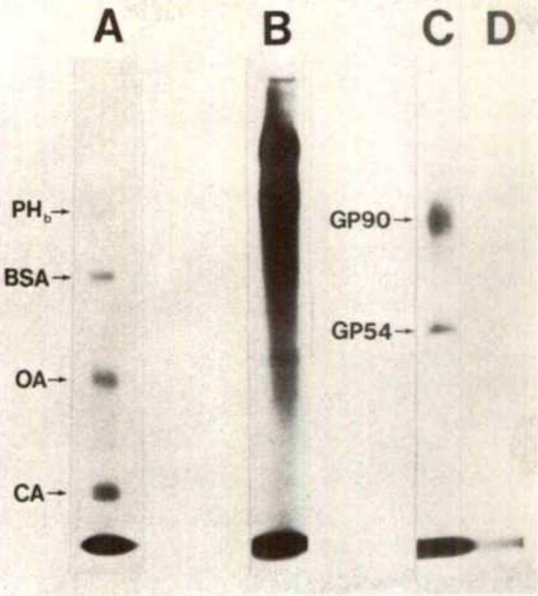

Fig 4. Polyacrylamide slab gel electrophoretic patterns of granulocyte surface glycoproteins reacting with anti-SP$_1$ antiserum. (A) Pattern of ^{14}C-labeled standard proteins: PH$_b$, phosphorylase b; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase (B) Triton X-100 lysate is presenting the total surface-labeled glycoproteins of granulocytes. (C) Pattern of immunoprecipitate obtained with anti-SP$_1$ antiserum from surface-labeled granulocytes. (D) Pattern of immunoprecipitate obtained with anti-SP$_1$ antiserum from surface-labeled granulocytes in the presence of 10 μg unlabeled SP$_1$. GP90, surface glycoprotein with an apparent mol wt of 90,000; GP54, surface glycoprotein with an apparent mol wt of 54,000.

When lysates from TPA-treated ML-2 cells, metabolically labeled with ^{35}S-methionine, were immunoprecipitated with anti-SP$_1$, two bands of 80,000 and 90,000 apparent mol wt were seen (Fig 6 F). The lower band was enriched on lentil lectin-Sepharose columns (Fig 6 L), which shows its glycoprotein nature. The two bands are apparently not components of a heterodimeric molecule linked by disulfide bridges, since a similar polyacrylamide gel pattern was obtained under nonreducing conditions (not shown).

DISCUSSION

The SP$_1$ glycoprotein belongs to the family of glycoproteins that show elevated serum levels during pregnancy. Placental tissue has been shown to synthesize this protein in vitro and is therefore considered as the main source of the SP$_1$ during pregnancy. In this study we have shown that the SP$_1$ glycoprotein is present in blood leukocytes and constitutes a late differentiation marker for the myelomonocytic lineage. Although most of the antigen, as judged by immunoreactivity, is located intracytoplasmically, a surface expression can also be observed that allows positive selection of the later stages of the granulocyte lineage by fluorescent-activated cell sorting.

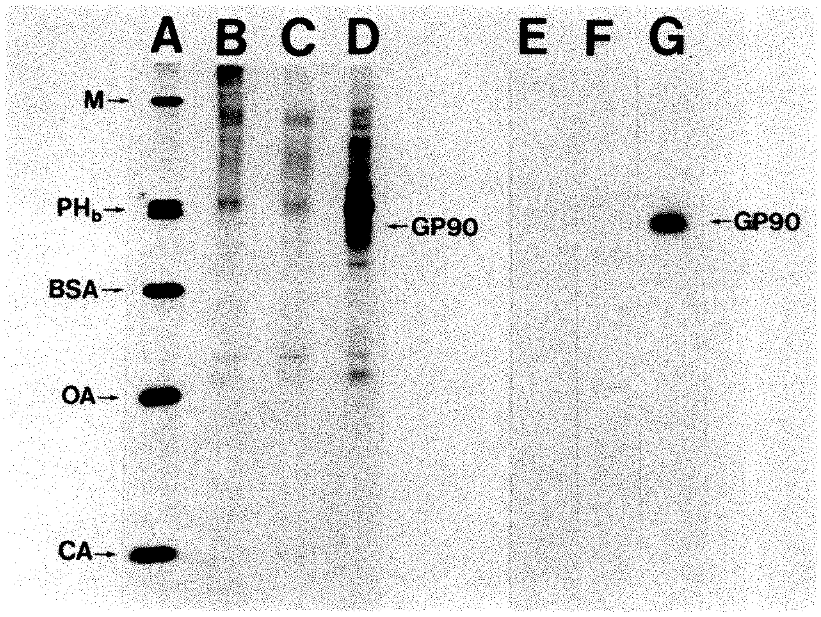

Fig 5. PAGE patterns of ML-2 cell surface glycoproteins reacting with anti-SP$_1$ antiserum. (A) Pattern of ^{14}C-labeled standard proteins, see legend to Fig 4; M, myosin. (B) Surface-labeled glycoproteins of untreated cells. (C) Surface-labeled glycoproteins of cells treated with retinoic acid. (D) Surface-labeled glycoproteins of cells treated with TPA. Twenty-five microliters of 1-mL lysates from 50 × 10^6 cells were loaded on B, C and D. (E) Immunoprecipitate obtained with anti-SP$_1$ antiserum from untreated cells. (F) Immunoprecipitate obtained with anti-SP$_1$ antiserum from cells treated with retinoic acid. (G) Pattern of immunoprecipitate obtained from cells treated with TPA.

The surface expression of SP$_1$ on granulocytes could further be substantiated by immunoprecipitation from lysates of surface-labeled, purified granulocytes. Analysis of the antigen precipitated by the anti-SP$_1$ antiserum on PAGE revealed a single major band of 90,000 apparent mol w. This corresponds to the mol wt reported for SP$_1$ of placental origin. Antigenic identity of the membrane protein in granulocytes and the SP$_1$ occurring in serum during pregnancy could be confirmed, since the addition of highly purified placental SP$_1$ could completely inhibit the immunoprecipitation with anti-SP$_1$ antiserum from surface-labeled granulocytes.

The availability of hematopoietic cell lines that phenotypically represent clonal expansions of certain lineages arrested at different stages of differentiation offers a useful tool for studies on biosynthetic events that are difficult to perform on limited amounts of available bone marrow cells. In this study we used a panel of hematopoietic cell lines that can be induced to further differentiation in vitro. Of these the promyelocytic cell line HL-60 and the myeloleukemia cell

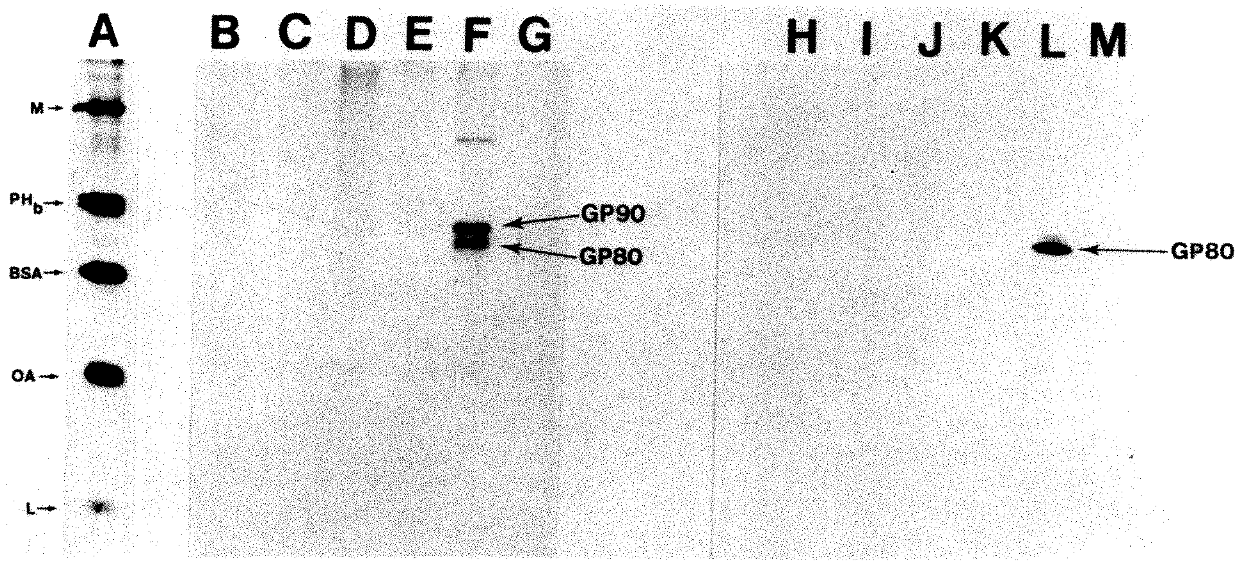

Fig 6. Polyacrylamide slab gel electrophoresis pattern of immunoprecipitates obtained with anti-SP$_1$ antiserum from ^{35}S-methionine-labeled ML-2 cells. (A) Patterns of ^{14}C-labeled standard proteins, see legend to Fig 4; L, lysosome. (B) Pattern obtained with antiserum directly from uninduced cells. (C) Control with nonimmune serum. (D) Pattern obtained with antiserum directly from retinoic acid-induced cells. (E) Control with nonimmune serum. (F) Pattern obtained with antiserum directly from TPA-induced cells. (G) Control with nonimmune serum. (H-M) Patterns obtained by immune precipitation from sugar eluates of lentil-lectin Sepharose columns. (H), With antiserum from uninduced cells. (I) Control with nonimmune serum. (J) With antiserum from retinoic acid-induced cells. (K) Control with nonimmune serum. (L) With antiserum from TPA-induced cells. (M) Control with nonimmune serum all from the same amount of lysates.

line ML-2 expressed appreciable amounts of SP$_1$ after induced differentiation. The pluripotent leukemia cell line K-562 which, like the HEL line, has capacity of erythroid differentiation, and the lymphoid lines remained negative before and after induced differentiation.

As TPA treatment in vitro of ML-2 induced the most dramatic increase in the expression of SP$_1$, this model was selected for further investigations. As seen for differentiating granulocytes in normal bone marrow and for monocytes in culture, induction of ML-2 cell differentiation led to a strongly increased expression of SP$_1$ intracytoplasmically, but reactivity was also found with the cell surface. Metabolic labeling of the TPA-induced ML-2 cells by ^{35}S-methionine followed by immunoprecipitation and PAGE revealed two closely spaced bands of 90,000 and 80,000 apparent mol wt, respectively. The two bands did not represent components of a heterodimeric molecule linked by disulfide bridges, since two distinct similar bands were obtained by gel electrophoresis under nonreducing conditions. Immunoprecipitation from surface labeled ML-2 cells, on the other hand, revealed only a single band of 90,000 mol wt corresponding to that of higher mol wt obtained from metabolically labeled cells. It is possible that SP$_1$ occurs in two forms in hematopoietic cells where the intracytoplasmic protein has a slightly lower apparent mol wt. Alternatively, the 80,000-mol wt band observed after metabolic labeling might represent an incompletely glycosylated precursor of the membrane-expressed 90,000-mol wt antigen. Pulse-chase experiments with TPA-induced ML-2 cells are now in progress to distinguish between these two possibilities.

TPA is commonly used in induction of differentiation both of hematopoietic and nonhematopoietic cells. The mechanism of action of this compound is still incompletely understood. The ability of TPA to activate protein kinase C might be involved in the transduction of the differentiation signals. We have earlier shown that treatment of ML-2 cells with TPA induces distinct alterations in the surface glycoprotein pattern.[16] While untreated ML-2 cells contain hardly detectable amounts of SP$_1$, treatment with TPA led to a strong increase in the synthesis of this glycoprotein. This phenomenon offers a new model for studies on the TPA-induced regulation of gene expression.

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Effect of Flow on Polymorphonuclear Leukocyte/Endothelial Cell Adhesion

By M.B. Lawrence, L.V. McIntire, and S.G. Eskin

The effect of flow on the adhesion of polymorphonuclear leukocytes (PMNL) to vascular endothelium was investigated using a parallel plate chamber with a well-defined flow field. Washed PMNL were perfused over a monolayer of primary human umbilical vein endothelial cells (HUVEC) pretreated with formyl-methionyl-leucyl-phenylalanine (FMLP, 1 × 10^{-7} mol/L) for five minutes. In other experiments HUVEC were pretreated with interleukin 1 (IL1, 2 U/mL) for four hours. PMNL adhesion to stimulated and control HUVEC was measured over a physiologic range of wall shear stresses. PMNL adhesion to nylon-coated surface was also studied. At a wall shear stress of 0.98 dynes/cm^2, 283 ± 37.3 PMNL/mm^2 (mean ± SEM) adhered to FMLP-treated HUVEC while 195 ± 20.3 PMNL/mm^2 adhered to control HUVEC. At 1.96 dynes/cm^2, 68 ± 14.1 PMNL/mm^2 adhered to FMLP-treated HUVEC and 42 ± 6.0 PMNL/mm^2 adhered to control HUVEC. At 3.92 dynes/cm^2, virtually no PMNL adherence was noted on either control or FMLP-treated HUVEC. On IL 1-treated HUVEC at 1.96 dynes/cm^2, 371 ± 25.8 PMNL/mm^2 adhered while 28 ± 2.9 PMNL/mm^2 adhered to control HUVEC. PMNL adhesion to IL 1-treated and control HUVEC dropped to 10.2 ± 3.8 and 6.8 ± 3.5 PMNL/mm^2, respectively, at 3.01 dynes/cm^2. The effect of flow on PMNL adhesion appears to be an important factor in determining the outcome of the PMNL/HUVEC adhesive interaction under these experimental conditions.

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ONE OF THE FIRST responses of the immune system to injury or infection is the margination of leukocytes to the blood vessel wall. While the effects on leukocytes and endothelium of various inflammatory mediators have been extensively studied under quiescent conditions, little work has been done in characterizing the magnitude and significance of physical forces on the process of leukocyte attachment to the vessel wall. With the well-defined flow field that exists in a parallel-plate flow chamber, the importance of wall shear stress in the margination of polymorphonuclear leukocytes (PMNL) can be quantitated with respect to the effects of inflammatory mediators.

A number of recent investigations have pointed to an independent role for the endothelium in mediating PMNL adhesion. Chemotactic compounds such as leukotriene B_4, complement fragment C5a, and formyl-methionyl-leucyl-phenylalanine (FMLP) in nanomolar concentrations have been shown in several laboratories to increase adhesion of PMNL to endothelium, apparently by action on the endothelial cell (EC).[1-3] In one set of experiments we used the bacterial endotoxin analog, FMLP, to stimulate adhesion of PMNL to endothelium. Perhaps the most dramatic modulators of the adhesive interaction now being investigated are interleukin 1 (IL 1), tumor necrosis factor, thrombin, and lipopolysaccharide. These agents induce large increases in leukocyte adhesion to EC and, in the cases of IL 1 and thrombin, appear to act selectively on the EC.[4-8] In a related set of experiments we used monocyte-derived IL 1 to stimulate the endothelium and measured PMNL adhesion to endothelium under varying flow conditions. Our data show a strong flow dependence of PMNL adhesion to endothelium that modulates the previously reported chemical effects of FMLP and IL 1.

One of the more interesting characteristics of PMNL observed in various tissue preparations is their ability to roll along the endothelium of postcapillary venules for considerable distances before detaching, stopping, or emigrating through the blood vessel wall.[9-12] This report presents measurements of PMNL rolling velocities on control and FMLP-treated endothelium at varying wall shear stresses.

It has been postulated that granulocytes play a significant role in clinical complications resulting from extracorporeal blood flow, such as occurs during hemodialysis and cardiopulmonary bypass procedures.[13-15] The transient neutropenia and pulmonary sequestration of leukocytes associated with these procedures are thought to be caused by the activation of complement by blood-contacting surfaces.[13,15-17] We report data on PMNL adhesion to nylon surfaces after exposure to human plasma under conditions in which the likely mediator of the adhesive interaction is surface-bound complement fragment C3b.

MATERIALS AND METHODS

Endothelial cell cultures. Human umbilical vein endothelial cells (HUVEC) were harvested using collagenase digestion,[18] pooled, seeded onto glass slides (34 × 74 mm, Fisher Scientific Co, Springfield, NJ), and cultured in M199 (GIBCO Laboratories, New York) supplemented with 20% fetal calf serum (FCS, HyClone Laboratories Inc, Logan, UT), penicillin, streptomycin, and neomycin (PSN, 1% by volume, GIBCO). Cultures were used in experiments two to four days after reaching confluence.

Preparation of human PMNL. Venous blood from normal, healthy male donors was drawn into heparin (10 U/mL, final concentration). PMNL were isolated from heparinized whole blood by dextran sedimentation followed by hypotonic lysis of erythrocytes and centrifugation over Ficoll-Hypaque (FH).[19,20] The final pellet was resuspended in M199 at pH 7.4 to which 3.75 mg/mL of bovine

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serum albumin (BSA, Sigma Chemical Co, St. Louis) was added (M199+BSA). PMNL were counted with a model ZBI Coulter Counter (Coulter Electronics Inc, Hialeah, FL) connected to a Coulter Channelyzer (Coulter Electronics) to determine the appropriate dilution ratio. Trypan blue exclusion showed greater than 96% viability.

Preparation of platelet-poor plasma. Venous blood was drawn into siliconized glass tubes containing heparin (10 U/mL, final concentration). After centrifugation at 400 × g for ten minutes, the supernatant was drawn off with a Pasteur pipet and centrifuged again at 1,000 × g for ten minutes. The resultant platelet-poor plasma (PPP) was transferred to a clean plastic tube.[20]

Experimental apparatus. To produce a well-defined wall shear stress, we used a parallel plate geometry for our flow chamber (Fig 1A).[21-24] One side of the chamber was a slide on which HUVEC were cultured. The other side was machined from polycarbonate. Once assembled these two flat surfaces were held approximately 250 μm apart by a Silastic rubber gasket (Dow Corning, Midland, MI). Because of the small gap-to-width ratio, the channel could be approximated over most of its surface as two infinite parallel plates. Under laminar flow conditions such as used in our experiments (Reynolds number < 2), the velocity profile is parabolic and the wall shear stress can be calculated utilizing the momentum balance for a Newtonian fluid.[24] As described in the following equation, the wall shear stress is equal to:

$$\tau = \frac{3\mu Q}{2a^2 b}$$

τ = Wall shear stress

μ = Coefficient of viscosity

Q = Volumetric flow rate

2a = Channel height

b = Channel width

A model 935 Harvard Apparatus syringe pump (South Natick, MA) was employed for flow control. Experiments were conducted at the following wall shear rates: 140 sec^{-1}, 280 sec^{-1}, 430 sec^{-1}, and 560 sec^{-1}. These shear rates, for a flowing tissue culture medium at 37°C, correspond respectively to wall shear stresses of 0.98, 1.96, 3.01, and 3.92 dynes/cm^2. This range of shear stresses is similar to those seen in venules and veins. PMNL/HUVEC interactions under these flow conditions were visualized directly using phase-contrast videomicroscopy and digital image processing (Fig 1).

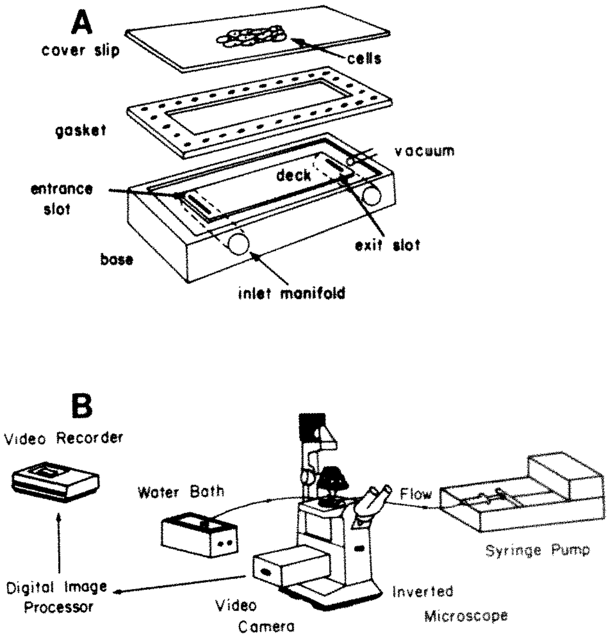

Fig 1. (A) Parallel plate flow chamber machined from transparent polycarbonate. The cover slip is covered with confluent HUVEC or coated with nylon. The silastic gasket separates the cover slip from the deck of the flow chamber and, when vacuum is applied, results in a channel gap of 250 μm. (B) The flow chamber is attached to the stage of the inverted-stage microscope. The syringe pump controls the flow and the water bath containing the PMNL cell suspension. An air-curtain incubator maintains the flow chamber, inlet tubing, and microscope stage at 37°C. The output of the video camera is recorded for later image processing and analysis.

Adhesion assay. The HUVEC covered slide was fitted onto the flow chamber and vacuum applied to hold it in place. The chamber was then rinsed with M199+BSA at 37°C for 30 minutes. The PMNL suspension was diluted with M199+BSA at 37°C to a cell concentration of 1,000 cells/μL and 30 minutes was allowed for the PMNL to stabilize. The PMNL suspension, the connecting tubing, and the microscope with flow chamber (Fig 2 B) were all maintained at 37°C by an air-curtain incubator (Laboratory Products, Boston; Model 279). Approximately 30 minutes after the PMNL were diluted, the HUVEC were exposed to 1 × 10^{-7} mol/L FMLP for five minutes and then rinsed for five minutes with M199+BSA. The PMNL suspension was then perfused through the flow chamber. Using a Hamamatsu video camera (Waltham, MA; Model C1012) mounted on a Nikon Diaphot-TMD inverted phase-contrast microscope (Garden City, NY), the experiments were videotaped. The number of PMNL attached to the HUVEC monolayer were counted after ten minutes of flow. The control consisted of the same experimental procedure without the incubation of the HUVEC with FMLP. For experiments involving IL 1 (Genzyme Inc., Boston), 100 μL of IL 1 (200 U/mL) in 0.5% BSA was introduced into the petri dish containing the HUVEC monolayer and diluted in the tissue culture medium to 2 U/mL. The HUVEC were incubated with the IL 1 for four hours preceding the experiment.[5] The control consisted of untreated HUVEC. The PMNL for each experiment were isolated from a single donor, and the HUVEC monolayers were from the same pooled seeding.

PMNL rolling velocities were measured by digital image processing (Perceptive Systems Inc., Houston; Model 327) of videotapes. The time interval used for these velocity measurements was 30 seconds to resolve PMNL velocities below 1 μm/sec. Accordingly, a stable adhesion was defined as one that lasted 30 seconds or longer. Normalized histograms of velocity distributions and the averages were determined from analyzing two to four frames of videotape separated by at least one minute in each experiment.

The same procedure for PMNL preparation was used with studies of PMNL adhesion to nylon. Glass slides were coated with Elvamide nylon (DuPont, Wilmington, DE) by solvent casting (MeOH, 1% solution). In some experiments the nylon surface was incubated with PPP for 20 minutes followed by a two-minute washout before the PMNL suspension was perfused through the chamber.

Statistical analysis. The variation in adhesion ratios was analyzed using a one-tailed *t* test for paired data since the control, FMLP-treated and IL 1-treated HUVEC, were from the same population of pooled cells, and the washed PMNL used in the experiment were from the same donor. Ratios of the means of PMNL rolling velocities on FMLP-treated HUVEC and controls as well as ratios of the means at two shear stresses were compared using a two-tailed *t* test. A two-tailed *t* test was also used to compare PMNL rolling velocity distributions. Error bars on all histograms represent the SEM.

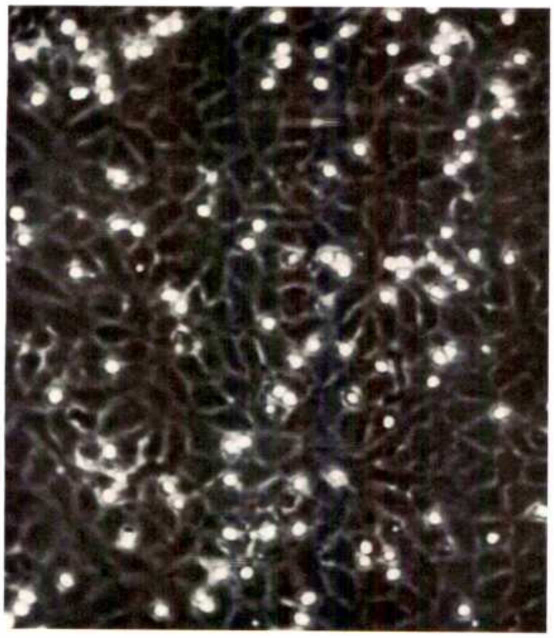

Fig 2. PMNL adherent to FMLP-treated HUVEC monolayer under flow taken from digitized frame of videotape (magnification 20 × objective). The large white dots are PMNL adhering to the HUVEC monolayer (dark background with barely visible HUVEC outlines). PMNL counts in the field of view are normalized to PMNL/mm^2. Wall shear stress = 0.98 dynes/cm^2. (Figure reduced to 65% of original size.)

RESULTS

In paired experiments at 0.98 dynes/cm^2 wall shear stress, the ratio of PMNL adhesion to FMLP-treated HUVEC *v* control HUVEC was 1.45 ($P < .01$, n = 9, Fig 3). At this wall shear stress 283 ± 37.3 PMNL/mm^2 (mean ± SEM) adhered to FMLP-treated HUVEC and 195 ± 20.3 PMNL/mm^2 adhered to control HUVEC after ten minutes of flow. At 1.96 dynes/cm^2 wall shear stress, the ratio of PMNL adhesion to FMLP-treated HUVEC *v* control HUVEC was 1.58 ($P < .025$, n = 8). At this wall stress 68 ± 14.1 PMNL/mm^2 adhered to FMLP-treated HUVEC and 42 ± 6.0 PMNL/mm^2 adhered to control HUVEC after ten minutes of flow. At 3.92 dynes/cm^2 wall shear stress, virtually no cell adherence was noted on either control or FMLP-treated HUVEC (<1 cell/mm^2, n = 3), although there were many transient white cell/endothelial cell interactions.

In paired experiments at 1.96 dynes/cm^2 wall shear stress, 371 ± 25.8 PMNL/mm^2 adhered to IL 1-treated HUVEC and 28 ± 2.9 PMNL/mm^2 adhered to control HUVEC, resulting in an adhesion ratio of 13.8 ± 1.2 ($P < .01$, n = 5, Fig 4). At 3.01 dynes/cm^2 wall shear stress, 10.2 ± 3.8 PMNL/mm^2 adhered to IL 1-treated HUVEC and 6.8 ± 3.5 PMNL/mm^2 adhered to control HUVEC, giving an adhesion ratio of 1.65 ± 0.25 ($P < .05$, n = 5).

At 0.98 dynes/cm^2 wall shear stress, 9.7 ± 3.8 PMNL/mm^2 adhered to the nylon surface while on nylon pretreated with PPP, 77 ± 21.7 PMNL/mm^2 adhered, giving an adhesion ratio of 7.9:1 ($P < .01$, n = 4, Fig 5). There was almost no adhesion (<1 cell/mm^2, n = 3) of PMNL to untreated nylon at 1.96 dynes/cm^2 (data not shown).

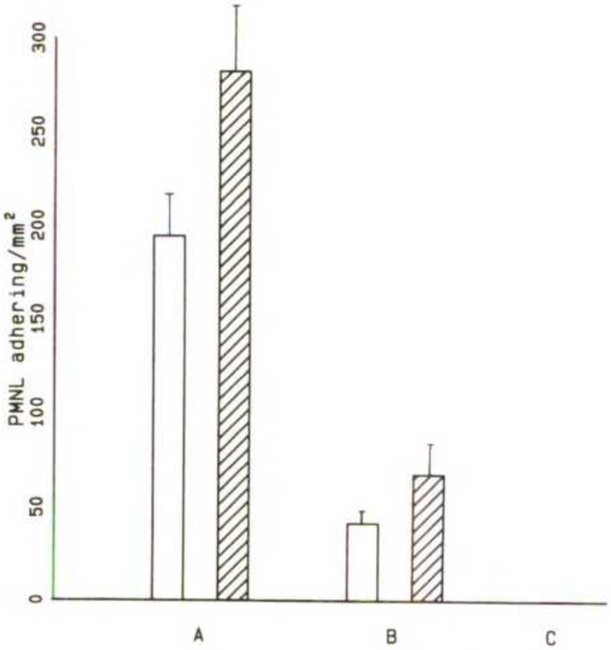

Fig 3. Effect of flow and FMLP-treatment of HUVEC on PMNL adhesion. Experiments performed at wall shear stresses of 0.98 dynes/cm^2 (A, 140 sec^{-1}), 1.96 dynes/cm^2 (B, 280 sec^{-1}), and 3.92 dynes/cm^2 (C, 560 sec^{-1}). PMNL were suspended in M199+BSA (1,000 cells/μL) at 37°C. FMLP-treated HUVEC (1 × 10^{-7} mol/L, five minutes), cross-hatched bars; control HUVEC, open bars. Error bars represent SEM.

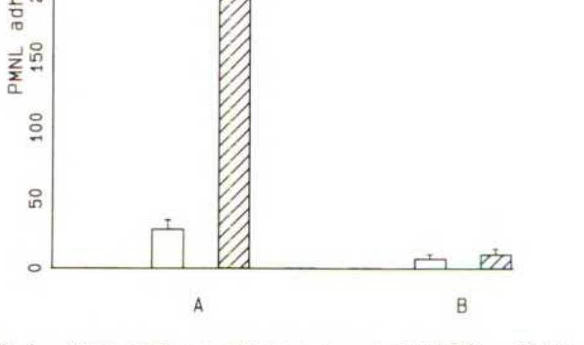

Fig 4. Effect of flow and IL 1 treatment of HUVEC on PMNL adhesion. Experiments performed at wall shear stresses of 1.96 dynes/cm^2 (A, 280 sec^{-1}) and 3.01 dynes/cm^2 (B, 430 sec^{-1}). PMNL were suspended in M199+BSA (1,000 cells/μL) at 37°C. IL 1-treated HUVEC (2 U/mL, four hours), cross-hatched bars; control HUVEC, open bars. Error bars represent SEM.

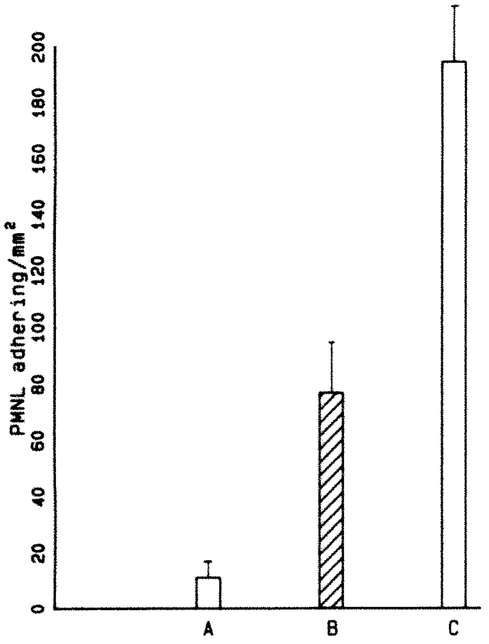

Fig 5. PMNL adhesion to nylon-coated surface under flow (0.98 dynes/cm², 140 sec⁻¹). PMNL were suspended in M199+BSA (1,000 cells/μL) at 37°C. PMNL/mm² adhering to untreated nylon surface (A), PMNL/mm² adhering to nylon preincubated with PPP for 20 minutes (B), PMNL/mm² adherent to control HUVEC (C). Error bars represent SEM.

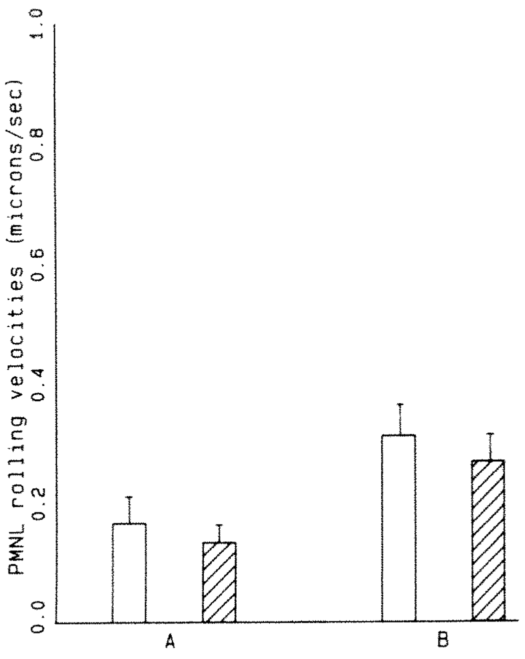

Fig 6. Effect of flow and FMLP treatment of HUVEC on the mean rolling velocities of PMNL. Experiments performed at wall shear stresses of 0.98 dynes/cm² (A, 140 sec⁻¹) and 1.96 dynes/cm² (B, 280 sec⁻¹). FMLP-treated HUVEC, cross-hatched bars; control HUVEC, open bars. Error bars represent SEM.

At a wall shear stress of 0.98 dynes/cm², PMNL that were attached to control HUVEC had an average rolling velocity of 0.166 ± 0.046 μm/sec; and PMNL attached to FMLP-treated HUVEC had an average rolling velocity of 0.134 ± 0.026 μm/sec (n = 3, Fig 6). The difference between these means due to FMLP treatment of the HUVEC monolayer was not significant ($P > .4$). At a wall shear stress of 1.96 dynes/cm², PMNL attached to control HUVEC had an average rolling velocity of 0.313 ± 0.054 μm/sec, and PMNL attached to FMLP-treated HUVEC had an average rolling velocity of 0.269 ± 0.047 μm/sec (n = 5). The difference between these means due to FMLP treatment of the HUVEC monolayer was also not significant ($P > .4$). The ratio of PMNL rolling velocities at 1.96 and 0.98 dynes/cm² wall shear stress was 1.94 ($P < .02$).

Between 20% and 90% of the PMNL with stable adhesions to the HUVEC monolayer rolled downstream. At 0.98 dynes/cm², less than 1% of the PMNL rolled faster than 1 μm/sec (Fig 7), while at 1.96 dynes/cm², approximately 5% of the PMNL rolled faster than 1 μm/sec (Fig 8). Differences in velocity distributions due to FMLP treatment of the

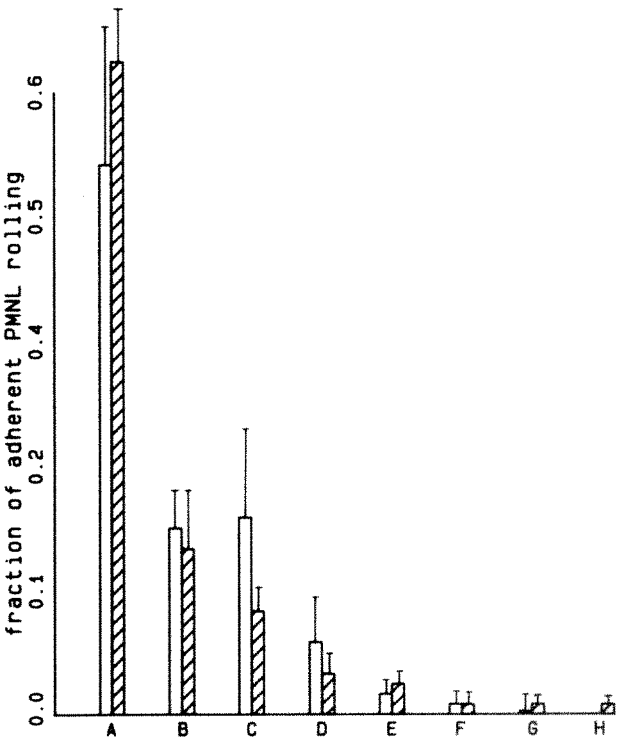

Fig 7. Distribution of PMNL rolling velocities at 0.98 dynes/cm². FMLP-treated HUVEC, cross-hatched bars; control HUVEC, open bars. Each pair of bars represents the fraction of adherent PMNL with rolling velocities ranging from 0.0 to 0.13 μm/sec (A), 0.13 to 0.26 μm/sec (B), 0.26 to 0.39 μm/sec (C), 0.39 to 0.52 μm/sec (D), 0.52 to 0.65 μm/sec (E), 0.65 to 0.78 μm/sec (F), 0.78 to 0.91 μm/sec (G), and 0.91 to 1.04 μm/sec (H). To generate a representative velocity distribution for each experiment, the normalized velocity distributions from two to four image frames of videotapes (separated in each case by at least one minute) were averaged. Histograms from experiments under the same conditions were again averaged to create a final normalized velocity distribution. Error bars represent SEM.

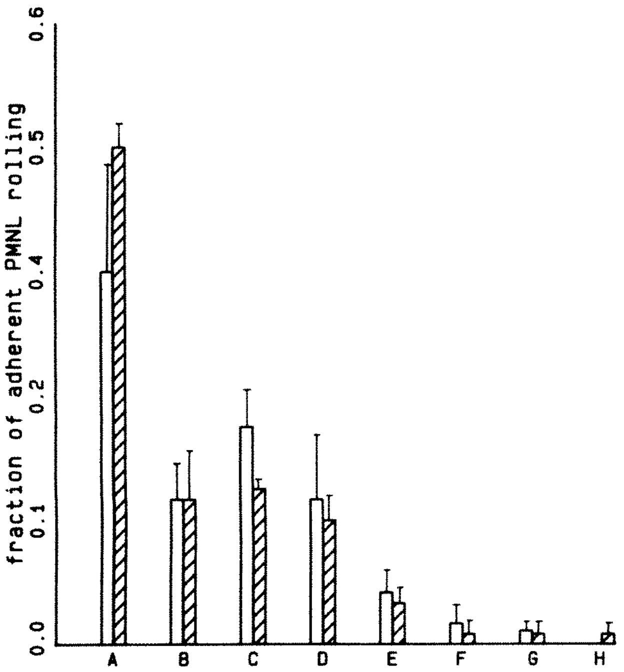

Fig 8. Distribution of PMNL rolling velocities at 1.96 dynes/cm^2. FMLP-treated HUVEC, cross-hatched bars; control HUVEC, open bars. Each pair of bars represents the fraction of adherent PMNL with rolling velocities ranging from 0.0 to 0.13 μm/sec (A), 0.13 to 0.26 μm/sec (B), 0.26 to 0.39 μm/sec (C), 0.39 to 0.52 μm/sec (D), 0.52 to 0.65 μm/sec (E), 0.65 to 0.78 μm/sec (F), 0.78 to 0.91 μm/sec (G), and 0.91 to 1.04 μm/sec (H). To generate a representative velocity distribution for each experiment, the normalized velocity distribution from two to four image frames of videotape (separated in each case by at least one minute) were averaged. Histograms from experiments under the same conditions were again averaged to create a final normalized velocity distribution. Error bars represent SEM.

HUVEC monolayer were not significant at the two flow conditions examined, although a broader distribution of rolling velocities was observed at 1.96 dynes/cm^2.

In experiments with nylon surfaces, we observed no PMNL downstream rolling, although PMNL in the plane of focus next to the nylon surface had velocities that ranged from 300 to 600 μm/sec. Extensive spreading of PMNL attached to the PPP-incubated nylon surface was observed.

DISCUSSION

From our experiments fluid drag forces appear to have a major impact on the number of PMNL adhering to endothelium. Doubling the wall shear stress from approximately 1 to 2 dynes/cm^2 reduced the number of adherent PMNL by more than a factor of four on both control and FMLP-treated endothelium. Doubling the wall shear stress again from approximately 2 to 4 dynes/cm^2 reduced adhesion to virtually zero. On IL 1-treated endothelium, the level of adhesion was 13-fold more than on controls at 2 dynes/cm^2, as was expected from earlier results of static experiments on PMNL/EC adhesion.[5] Increasing the wall shear stress to approximately 3 dynes/cm^2 on the IL 1-treated HUVEC dropped both the adhesion ratio and the absolute number of adherent PMNL dramatically. This large decrease in the level of PMNL adhesion, even on highly stimulated endothelium, over a small range of wall shear stresses is in accord with Lackie's proposal that the PMNL/EC adhesive force is delicately balanced with the shear force due to fluid drag.[25,26]

The wall shear rates examined in these experiments are close to those estimated for the venules and large blood vessels but are lower than those estimated for the capillary bed where there is normally little PMNL adhesion.[27] In vivo, the abrupt drop in shear forces found in the transition from capillaries to venules coincides with the location of the bulk of PMNL adhesion in the microcirculation.[26] A qualitatively similar pattern can be seen in our model vessel data using dilute suspensions of PMNL. Our data suggest that PMNL adhesion to endothelium (even activated endothelium) can occur only after shear forces have dropped below a threshold level and may explain in part why PMNL normally adhere in venules and not in capillaries.

By eliminating complex geometry and the resultant complex fluid mechanics, the flow system described simplifies the type of forces a PMNL experiences and permits a direct correlation of wall shear stress with the level of PMNL/EC adhesion. In our experiments a dilute, washed PMNL suspension was used so that any influence of platelets or erythrocytes would be eliminated. However, there has been some recent evidence that in plasma PMNL adhesion to EC is reduced.[28] Pretreatment of the PMNL with FMLP was not done, as the purpose of this work was to evaluate the role of the endothelial cell substrate on PMNL adhesion. Preactivation of PMNL would probably lead to very different levels of leukocyte adhesion to HUVEC. It has been shown that FMLP causes a 20-minute aggregation response in PMNL, so the incubation times for endothelial cells were selected accordingly.[20] Tonneson et al[29] have reported that exposure of endothelial cell monolayers to FMLP at a comparable concentration for periods of time ranging from 15 minutes to 4 hours did not increase PMNL adhesion to the monolayer. Thus, the small increases in adhesion seen in our work (Fig 3) and that of others may be due to some PMNL activation by low levels of residual FMLP.[1,3]

The number of PMNL sticking to nylon was very low when compared with the number of PMNL that adhered to endothelium at a wall shear stress of 0.98 dynes/cm^2. When the wall stress was doubled, we observed no PMNL adhering to the untreated nylon surface. The contact of the nylon-coated flow chamber wall with PPP, as would occur if blood were in contact with a similar type of foreign surface, dramatically increased the adhesion of PMNL[30] (Fig 5). PMNL have receptors for the complement fragments C3b and C3bi for effectively attaching to opsonized surfaces and facilitating the phagocytosis of bacteria.[31,32] However, when nylon activates the C3 convertase enzyme, the C3b and C3bi fragments formed probably bind to nylon's polysaccharide-like surface, possibly forming an "opsonized" surface for the PMNL to attach to.

An unattached PMNL near the wall of our flow chamber would be moving at a velocity of several hundred μm/sec if there were no interactions with the endothelial cell monolayer, as was the case of PMNL near the nylon surface. In contrast, the mean rolling velocities of PMNL we report on endothelium are considerably less than 1 μm/sec, which indicates that there is a strong adhesive interaction occurring. In addition, our data show a strong dependence of mean rolling velocity on wall shear stress. The mean rolling velocity of PMNL subjected to a wall shear stress of 1.96 dynes/cm^2 was approximately double the mean rolling velocity of PMNL subjected to 0.98 dynes/cm^2.

Atherton and Born[10] have reported that in vivo granulocyte rolling velocities exhibit a strong flow dependence. Unexpectedly, there was no significant difference in average PMNL rolling velocities due to FMLP-pretreatment of the HUVEC at either flow rate examined. With both FMLP and control experiments, the effect of increased wall shear stress was to broaden the distribution of velocities of rolling PMNL at the higher flow rate as well as to raise the mean rolling velocity of attached cells. The velocity distributions in all the experiments analyzed were similar and show consistently that 20% to 90% of the attached PMNL were rolling downstream. Since a large fraction of PMNL roll downstream due to flow, it is possible that this intermediate level of adhesion is a property of leukocytes that allows them to marginate from the bulk flow in the circulation yet still retain some mobility along the vessel wall. In our experiments with PMNL adhering to nylon, we observed what appeared to be cell spreading but no rolling PMNL, which suggests the interaction is of a different nature than the PMNL/HUVEC adhesive interaction.

Little is known about the type and strength of the bonds formed between PMNL and EC or the actual forces on the interface that determine whether or not the PMNL will adhere.[9,11] Employing a centrifugation assay that determined the force needed to separate PMNL from an endothelial cell monolayer after coincubation of the two cell types, Charo et al[33] have reported that FMLP and C5a increase the strength of PMNL adhesion to endothelium. A stronger adhesive interaction with the stimulated endothelium implies that rolling velocities would be lower, and PMNL flowing near the EC monolayer would be more readily arrested. However, only slight differences were observed between PMNL rolling velocities on FMLP-treated and control endothelium. Additionally, our data indicated the threshold shear stresses for PMNL adhesion to untreated, FMLP-treated, and IL 1-treated HUVEC were very close to each other. The pattern of PMNL adhesion to HUVEC at different shear stresses suggests that, while there were many more adhesive sites on IL 1 and FMLP-treated endothelium, the adhesive bonds formed were not necessarily stronger than on untreated HUVEC.

Even though static conditions in the blood rarely occur, most studies of leukocyte adhesion have been conducted in systems without controlled flow effects. These studies have revealed much about the biochemical interactions involved in leukocyte adhesion to endothelium but say little about the relative importance of different forms of activation under the wide range of physiologic conditions PMNL encounter in vivo. Our data, showing that slight changes in wall shear stresses can cause large changes in PMNL adhesion to both stimulated and unstimulated HUVEC, suggest that the local control of blood flow—perhaps by vessel dilation—may be as significant as the presence of inflammatory mediators in determining where the arrest of leukocytes occurs.

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Phorbol Ester Induces Interleukin-2 Receptor on the Cell Surface of Precursor Thymocyte Leukemia With no Rearrangement of T Cell Receptor β and γ Genes

By Yasuo Morishima, Yoshihisa Morishita, Koichi Adachi, Mitsune Tanimoto, Ryuzo Ohno, and Hidehiko Saito

The early event of thymocyte maturation has been analyzed using acute lymphoblastic leukemia (ALL) cells. A group of ALL cells whose cell surface phenotype was CD2 (SRBC receptor) negative and CD7 (T cell antigen) positive has been considered as precursor thymocyte ALL (pre-T-ALL). No rearrangements of the T cell receptor β-gene (TCR β) and γ-gene (TCR γ) were found in three of four pre-T-ALL patients. Stimulation of these pre-T-ALL cells with 12-0-tetradecanoylphorbol-13-acetate (TPA) induced only CD25 (Tac) antigen but no other T cell antigens. These findings suggest that the activation pathway of interleukin 2 (IL-2) receptor already exists in the most immature precursor thymocytes. Pre-T-ALL cells from the fourth patients showed the expression of CD3 antigen, and both TCR β and TCR γ rearrangement. TPA induced the differentiation of the more mature pre-T-ALL cells of this case in vitro, and not only CD25 (Tac) antigen but also CD4 and CD8 antigens appeared on the cell surface. The low affinity binding of ^{125}I-IL-2 to TPA-stimulated leukemia cells was observed in the three cases of pre-T-ALL tested, and the addition of recombinant IL-2 to TPA-stimulated cells showed no effect on cell proliferation.

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IT HAS RECENTLY become evident that the process of T cell differentiation from immature thymocytes to mature functional T cells accompanies the rearrangement of T cell receptor (TCR) genes and the sequential changes of T cell surface differentiation antigens.[1-4] Precursor T cells that migrate to thymus will acquire the potential to respond to exogenous stimulation during the activation process of TCR genes. These early events of T cell maturation in the thymus have been analyzed using acute lymphoblastic leukemia (ALL) cells, because some type of leukemia cells is considered to be a clonal expansion of thymocytes at certain stages of T cell differentiation.[5,6]

A group of ALL that is supposed to arise from immature precursor thymocytes has been identified by several investigators.[7-10] These ALL cells have the characteristic cell surface phenotype of CD7 positive and CD2 (sheep RBC [SRBC] receptor) negative. It was also reported that some of these ALL cells showed no TCR β gene rearrangement.[11,12]

In order to investigate the earliest event in the thymus, we selected CD7 (+) CD2 (−) ALL cells, and analyzed not only the cell surface antigens but also TCR β and γ gene rearrangements. Furthermore, these leukemia cells were stimulated with phorbol diester, 12-0-tetradeconoylphorbor-13-acetate (TPA), and the changes of T cell differentiation antigens were analyzed.

MATERIALS AND METHODS

Leukemia sample. Four patients with acute leukemia with CD7 positive and CD2 negative were analyzed. Case no. 1 was an 11-year-old boy with thymoma, splenomegaly, and hepatomegaly at diagnosis. Peripheral blood WBC count (PB-WBC) was 37,900/mm^3 with 90% leukemia cells, and >92% of bone marrow nucleated cells (BM-NC) were leukemia cells morphologically at diagnosis. Case no. 2 was a 23-year-old man without thymoma. PB-WBC was 22,000/mm^3 with 90% leukemia cells, and BM-NC contained 96% leukemia cells. Case no. 3 was a 9-year-old boy with thymoma. PB-WBC was 26,000/mm^3 with 80% leukemia cells and BM-NC contained 94% leukemia cells. Case no. 4 was a 19-year-old man without thymoma and with splenomegaly and hepatomegaly. PB-WBC was 300,000/mm^3 with 94% leukemia cells. BM-NC contained 97% leukemia cells. One patient with CD2 and CD7 positive T-ALL was also analyzed. All leukemia samples were obtained from bone marrow at diagnosis. The mononuclear cells were separated by Ficoll-Hypaque gradient method, and stored at −180°C in liquid nitrogen. After thawing, all of the samples had >90% viability and >95% leukemia cells. The Giemsa staining of these leukemia cells showed the morphological characteristics of ALL, and both peroxidase staining and esterase staining were negative in all cases.

Monoclonal antibody. Monoclonal antibodies (MoAbs) used for the analysis of cell surface antigens were NA1/34 (CD1), 9.6 and Leu 5b (CD2), UCHT-1 and T3 (CD3), T4 (CD4), UCHT-2 (CD5), 12.1 (CD6), 4A (CD7), Leu 2a (CD8), and T1A (CD25). CD number is the specificity of antigens according to the nomenclature of the International Workshop on Human Leukocyte Differentiation Antigens,[13] and all of these MoAbs were obtained from the second and the third workshops. MoAb 35.1 (CD2) for T cell purging was kindly provided by Dr J.A. Hansen, Fred Hutchinson Cancer Research Center, Seattle, and MoAb 4A (CD7) were produced and analyzed by Y. Morishima.[8]

Immunofluorescent test. The indirect immunofluorescent test (IF) was performed with saturating condition of MoAbs. Five × 10^5 cells were incubated with 50 μL of MoAbs at 4°C for 30 minutes. Excess antibody was removed by washing twice in phosphate-buffered saline (PBS) supplemented with 0.4% bovine serum albumin and 0.02% sodium azide. Then 50 μL of FITC-conjugated F(ab')$_2$ fragment of goat anti-mouse IgGM (1:40 dilution) (Tago, Burlingame, CA) was added, and the mixture was incubated for another 30 minutes at 4°C. The cells were further washed three times and the percentage of fluorescence positive cells was counted using flow cytometry (FACS analyzer, Beckton Dickinson, Mountain View, CA). Normal mouse serum was used as negative control for background fluorescence. The fluorescence positive cells of the samples were counted when the base line of negative control was set to be positive in <5% of cells.

Stimulation of leukemia cells with TPA. In order to remove the small amount of contaminated PB T cells in leukemia samples, 1 ×

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10^7 of precursor thymocyte ALL (pre-T-ALL) cell sample was incubated with 1 mL of MoAb 35.1 (100 times dilution of ascitis) for 30 minutes at 4°C, and baby rabbit complement (Pel-Freez, Rogers, AR) was then added at the final concentration of 50% (vol/vol). After 60 minutes of incubation at 37°C, the cells were washed twice, and used for the incubation with TPA (Paesel GmbH & Co, Frankfurt, West Germany). One × 10^6/mL of T cell-depleted leukemia cells in culture medium (RPMI 1640 with 10% fetal calf serum, penicillin and streptomycin) were incubated with 1×10^{-7} mol/L TPA at 37°C in a humidified 5% CO_2 incubator. After 2, 6, 12, 24, and 72 hours, the cells were harvested, and the changes of cell surface antigens on these stimulated cells were analyzed by IF test.

In another experiment, the proliferation of pre-T ALL cells by TPA with or without interleukin-2 (IL-2) was tested in triplicate. Two × 10^5/well of T cell-depleted leukemia cells were incubated with or without 1×10^{-7} mol/L TPA using a 96-well flat microplate at 37°C in a humidified 5% CO_2 incubator for 24 hours, and then recombinant IL-2, which was kindly provided from Takeda Pharmaceutical Co, Osaka, Japan, was added to the wells at a final concentration of 0.1 μg/mL. After two- and five-day incubation, 1 μCi of ^{3}H-thymidine (New England Nuclear, Boston) was added to the well, and the cells were cultured for another 15 hours. The incorporation of ^{3}H-thymidine into DNA was measured with liquid scintillation counter.

Radiolabeled IL-2 binding assay. Before determining the level of binding, cells were incubated twice for one hour at 37°C in 50 mL RPMI 1640 in order to remove endogeneous IL-2. After washing, the cells were resuspended in RPMI 1640 supplemented with 25 mmol/L Hepes (pH 7.3) and 10 mg/mL BSA (RPMI-BSA). Serial dilutions of recombinant human ^{125}I-IL-2 (32.6 μCi/ng, New Research Products, Boston) were incubated at 37°C for ten minutes with 5×10^5 cells in a total volume of 100 μL RPMI-BSA using a 96-well round bottomed microtiter plate. After incubation, the cells were centrifuged through a layer of silicon oil/paraffin oil mixture in 400 μL polyethylene tubes (9,000 g for 90 seconds) according to the method of Robb et al.[14] The supernatant from the first wash was counted to determine the level of unbound IL-2. After washing once, the tops of the tubes containing the cell pellet were cut off and were counted to determine the level of bound IL-2. The dissociation constant (kd) and the number of IL-2 receptor binding sites were calculated from Scatchard plot analyses.

Gene rearrangement analysis. High-molecular-weight DNA was prepared from frozen samples by standard sodium sulfate-proteinase K method followed by phenol extraction and ethanol precipitation. These DNA samples (15 μg) were digested with restriction enzymes *Bam*HI, *Eco*RI or *Hind*III (Boehringer Manheim GmbH, West Germany) and were size-separated by 0.7% agarose gel electrophoresis and were transferred by Southern's method[15] to nitrocellulose papers (Schleicher & Schuell, Keene, NH). These papers were then hybridized with nick-translated ^{32}P-labeled DNA probes at 42°C for 16 hours. Blots were washed at high stringency and were exposed against Kodak AR-5 film (Kodak, Rochester, New York).

Three probes used in this study were JH (1.2 Kb *Hind*III fragment of mouse heavy chain J region), which was kindly provided by Dr J. Ravetti, Memorial Sloan Kettering Cancer Research Institute, New York; C (0.7 Kb *Eco*RI fragment of mouse TCR β chain constant region), which was purchased from Oncor Co, Gaithersburg, MD; and Tγ5 (1.0 Kb *Eco*RI fragment of human TCR γ chain constant region), which was generously provided by Dr D. R. Littman, University of California.

RESULTS

Characterization of T cell differentiation antigens and TCR β, TCR γ, and JH gene rearrangement in pre-T-ALL cells. Four cases of pre-T-ALL with cell surface phenotypes of CD2 (−) and CD7 (+), and one case of T-ALL (case no. 5) were selected for analysis. All of four pre-T-ALL cells expressed another T cell antigen CD5, while only one case (case no. 4) expressed CD3 antigen, and no other T cell antigens (CD1, CD2, CD4, CD6, CD8) including Tac antigen (CD25) were detected on the cell surface of these 4 pre-T-ALL cells (Table 1).

Southern blot analysis of TCR β, TCR γ, and Ig heavy chain gene rearrangement demonstrated that no rearrangements of these three genes occurred in cases no. 1, 2, and 3, while TCR β and TCR γ gene had rearranged in cases no. 4 and 5 (Table 1). Restriction fragment patterns of TCR γ gene were shown in Fig 1. *Bam*HI digestion of germ line DNA (Fig 1, lane 7) resulted in 17-, 3.3-kb bands containing Cγ1 sequences and 12-, 5-, 4-kb bands containing Jγ2 to Cγ2 sequences, whereas *Eco*RI digestion of the DNA resulted in 5.0-, 2.2-kb bands containing Cγ1 and 6.9-, 1.8-kb bands containing Cγ2 regions. Distinct bands of 1.3 kb in *Eco*RI digests seem to represent fragments containing Cγ1 or Cγ2 sequences according to the restriction map of human genomic TCR γ chain gene (Dan R. Littman, personal communication). Three of four pre-T-ALL (cases no. 1, 2, and 3; Fig 1: lanes 2, 5, and 4, respectively) showed germ line pattern in both *Bam*HI and *Eco*RI digests, while case no. 4 of pre-T-ALL (lane 3) and case no. 5 of T-ALL (lane 6) exhibited the rearrangement of TCR γ gene involving Cγ2 region with the deletion of Cγ1 region.

Induction of Tac antigen (CD25) on pre-T-ALL cells by TPA stimulation. Pre-T-ALL cells were cultured with or without TPA, and the expression of various T cell antigens on

Table 1. Cell Surface Phenotype and Gene Rearrangement of Pre-T-ALL Cells

Cell Surface Antigen[*]	Monoclonal Antibody	Pre-T-ALL Case 1	Case 2	Case 3	Case 4	T-ALL Case 5
CD7	4A	81[†]	57	92	82	73
CD2	9.6	n[‡]	n	n	n	79
CD5	UCHT-2	19	21	78	62	61
CD3	UCHT-1	n	n	n	65	24
CD4	T4	n	n	n	n	26
CD8	Leu2a	n	n	n	n	17
CD6	12.1	n	n	n	n	n
CD1	NA1/34	n	n	n	n	n
CD25	T1A	n	n	n	n	n
Gene	Restriction Enzyme					
TCR β	BamHI	G	G	G	R	R
	EcoRI	G	G	G	R	R
TCR γ	EcoRI	G	G	G	D	D
	HindIII	G	G	G	D	D
	BamHI	G	G	G	R	R
JH	BamHI	G	G	G	G	G
	EcoRI	G	G	G	G	G

Abbreviation: G, germ line; R, rearrangement; D, deletion.

[*] Designation of the International Workshop on Human Leukocyte Differentiation Antigens.[13]

[†] Percent of indirect immunofluorescence (IF) positive cells by FACS analyzer.

[‡] IF negative, <10% of positive cells.

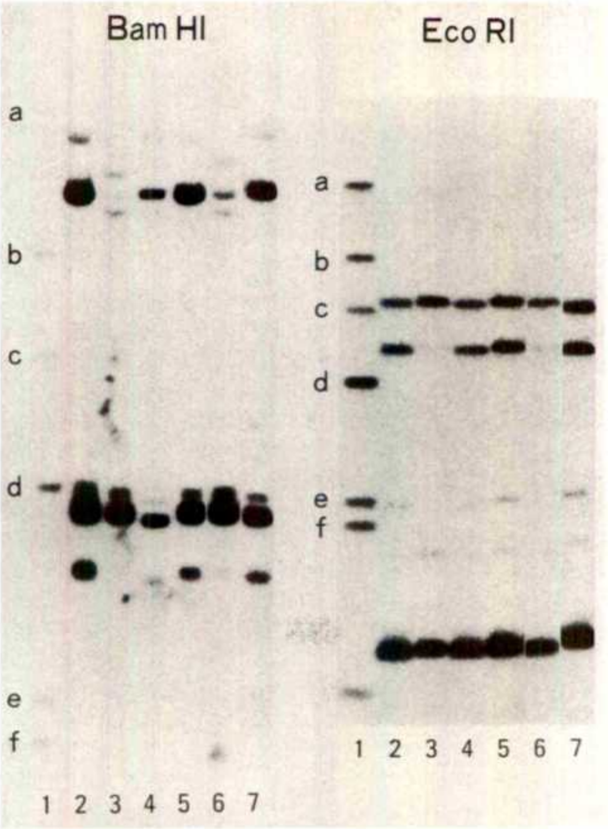

Fig 1. TCR γ gene rearrangement of pre–T-ALL cells. Southern blot analysis using a probe for TCR constant region was performed. (a-f) the size of standard fragment of DNA *Hind*III (a, 23 kb; b, 9.4 kb; c, 6.6 kb; d, 4.2 kb; e, 2.3 kb; f, 2.0 kb). Lane 1, standard; lane 2, case no. 1 (pre–T-ALL); lane 3, case no. 4 (pre–T-ALL); lane 4, case no. 3 (pre–T-ALL); lane 5, case no. 2 (pre–T-ALL); lane 6, case no. 5 (T-ALL); and lane 7, normal granulocytes.

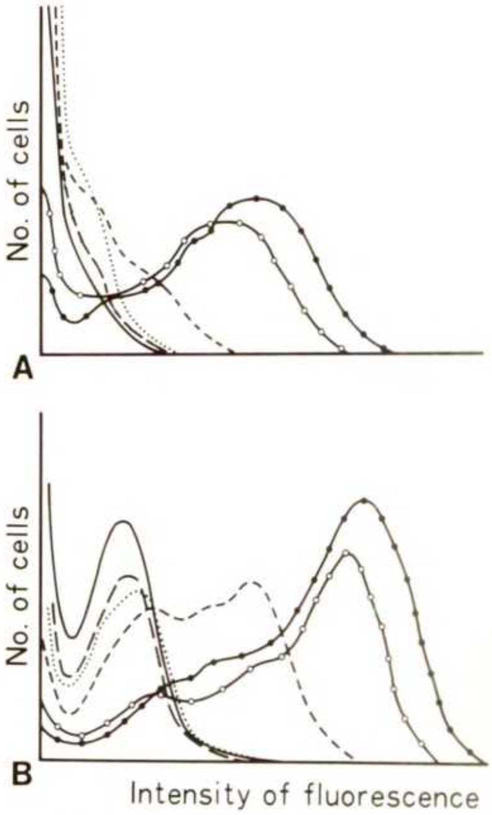

Fig 2. Changes of the expression of Tac antigen on pre–T-ALL cells by the stimulation of TPA. Pre–T-ALL cells were incubated with TPA, and indirect immunofluorescent test was performed using MoAb T1A (anti Tac, CD25), and analyzed by cytofluorography. The following lines indicate the incubation time: Before incubation, (—); after two hours, (– – –); six hours, (------); 12 hours (- - - - - -); 24 hours, (–○–○–); 72 hours (–●–●–).

these stimulated cells was analyzed by IF assay using fluorocytometry. The results after two-day culture were shown in Table 2. All four cases expressed Tac antigen (CD25) after TPA stimulation, while incubation without TPA for two days could not induce Tac antigen in all cases. The time course of the appearance of Tac antigen by TPA stimulation was analyzed in cases no. 1 and 4 (Fig 2). Tac antigen began to appear after 12 hours of incubation, and reached maximum expression after 24 hours in both cases. Increase in cell number during the culture was not observed, and the viability of the sample was kept at >80%.

Changes of the other T cell antigens on pre–T-ALL cells by TPA stimulation. As shown in Table 2, no CD2 antigen (SRBC receptor) appeared, and no CD1 or CD6 antigen was induced in all four cases (data not shown). Both CD7 antigen and CD5 antigen, which had been already expressed on the unstimulated cells, showed a tendency to decrease the per-

Table 2. Changes of T Cell Surface Antigens on Pre–T-ALL Cells by the Incubation With TPA

		Case 1			Case 2			Case 3			Case 4		
			TPA†			TPA			TPA			TPA	
Antigen	Monoclonal Antibody	Before*	(+)	(−)	Before	(+)	(−)	Before	(+)	(−)	Before	(+)	(−)
CD25	T1A	n‡	34	n	n	25	n	n	26	n	n	59	n
CD2	9.6	n	n	n	n	n	n	n	n	n	n	n	n
CD7	4A	81	15	55	56	n	45	92	73	46	82	57	56
CD5	UCHT-2	20	10	14	21	7	25	78	61	46	62	58	42
CD3	UCHT-1	n	n	n	n	n	n	n	28	n	65	42	40
CD4	T4	n	n	n	n	n	n	n	n	n	n	21	n
CD8	Leu2a	n	n	n	n	n	n	n	n	n	n	54	n

*Percent of IF positive before incubation.

†Percent of IF positive after two days of incubation with or without TPA.

‡n, <10% of positive cells.

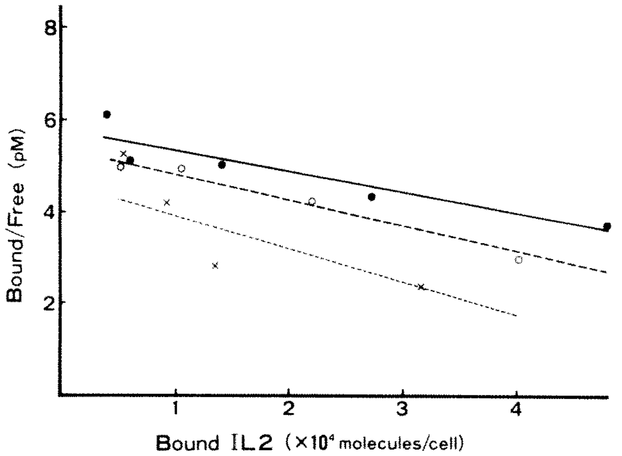

Fig 3. A Scatchard plot for the binding of ^{125}I-IL-2 to TPA-stimulated (24 hours) pre-T-ALL cells. Five × 10^5 cells were incubated with several dilutions of ^{125}I-IL-2. The data were corrected for nonsaturable binding by measuring the residual binding in the presence of 100-fold unlabeled IL-2 in selected tubes to give specific binding. Case no. 1, (●——●); case no. 2, (O----O); case no. 3, (x-----x).

centage and intensity of IF positive cells during the in vitro incubation with and without TPA. Interestingly, case no. 4 leukemia cells expressed CD4 and CD8 antigens by the stimulation with TPA for two days.

^{125}I-IL-2 binding analysis of TPA-incubated leukemia cells. For the further evaluation of the functional state of IL-2 receptor, which had been detected immunologically by anti-Tac antibody, various concentrations of ^{125}I-IL-2 were incubated with leukemia cells that had been treated with 1×10^{-7} mol/L TPA for 24 hours. The results of Scatchard plot analysis of such binding experiments confirmed that at IL-2 concentration of >1 nmol/L, saturable binding observed in both CD3(+) and CD3(−) pre-T-ALL cells after the stimulation with TPA (Fig 3). Low-affinity binding sites of CD3(−) pre-T-ALL were calculated to be 1.21×10^5/cell with kd 3.1 nmol/L (case no. 1) and 1.09×10^5/cell with kd 12.9 nmol/L (case no. 2), and those of CD3(+) pre-T-ALL (case no. 4) were 5.86×10^4/cell with kd 14.1 nmol/L.

Proliferative responses of pre-T-ALL cells to TPA and IL-2. In order to investigate whether pre-T-ALL cells that express IL-2 receptor by TPA stimulation respond to exogeneous IL-2, PB-T cell-depleted leukemia cells were cultured with TPA in the presence or absence of recombinant IL-2, and the proliferative responses of leukemia cells were measured by the uptake of ^{3}H-thymidine in case no. 1 and case no. 4 (Table 3). The increase of ^{3}H-thymidine uptake was observed in case no. 4 with the stimulation of TPA alone, and a maximum uptake was observed at four days of incubation. On the other hand, the uptake with the stimulation of TPA remained at the same level as that without TPA in case no. 1. When exogeneous IL-2 was added to the TPA-stimulated leukemia cells, no additional increase of ^{3}H-thymidine uptake was observed in either case tested.

DISCUSSION

ALL with the cell surface antigens of negative SRBC receptor (CD2) and positive CD7 T cell antigen has been classified as one category of ALL by Haynes using MoAb 3A1 (CD7),[7,16] Morishima et al using MoAb 4A (CD7),[8] Link et al using MoAb 4H9 (CD7),[9] and Vodinelich et al using MoAb WT1 (CD7).[10] There is evidence that these leukemia cells originate from precursor thymocytes as follows: (1) Many of these leukemia cells express not only CD7 T cell antigen, but also CD5 T cell antigen; (2) many of these leukemia patients have thymoma at diagnosis; and (3) these leukemia cells do not express Leu 7 and Leu 11 antigens, which are the marker of natural killer cells (data not shown). Furthermore, Tsuge et al's finding[17] that SRBC receptor-negative normal thymocytes do express CD7 antigen also supports the origin of this leukemia.

In the present study, three precursor thymocyte (pre-T) ALL cases, which expressed no T cell antigens except CD7 and CD5, showed no TCR β gene rearrangement. These data are in good agreement with Pittaluga et al,[11] and Suzuki et al[12] reports that CD7 expression precedes TCR β gene rearrangement in precursor T lymphoblastic neoplasms. As indicated by Pittaluga et al,[18] our three pre-T-ALL cases with no rearrangement of TCR β gene also showed no rearrangement of TCR γ gene. Therefore, these three pre-T-ALL had most immature characteristics of precursor thymocytes in which both TCR β and γ genes are kept in germ line configurations. Our preliminary data showed that all of three T-ALL cases and only one of four adult T cell leukemia cases also showed TCR γ gene rearrangement (data not shown). Griesser et al also reported that B cell lymphoma cells showed TCR γ gene rearrangement.[19] Therefore, TCR γ gene rearrangement is not always T cell specific, and the role of TCR γ gene rearrangement in pre-T-ALL cells will be examined until the function of TCR γ molecule[20] is elucidated.

Leukemia cells in case no. 4 expressed not only CD7 and CD5 antigens but also CD3 antigen, and both TCR β and γ gene rearrangement was observed. These data indicate that case no. 4 had more mature characteristics of thymocytes

Table 3. Effect of Exogeneous IL-2 on the Proliferation of TPA-Stimulated pre-T-ALL Cells

T-ALL Cell	Culture Period	Treatment*			
		TPA	TPA + IL-2	IL-2	None
Case 1	4 d	2974 ± 752	2909 ± 385	3960 ± 939	2925 ± 63
	7 d	1506 ± 146	2505 ± 268	4234 ± 875	2310 ± 210
Case 4	4 d	9144 ± 795	8604 ± 1102	905 ± 244	489 ± 135
	7 d	1513 ± 35	2118 ± 425	1015 ± 271	167 ± 34

*2 × 10^5 cells were incubated with or without TPA for 24 hours, and then recombinant IL-2 was added and incubated for three or six days.
†mean count per minute ± SD.

than the other three pre–T-ALL cases, and the leukemia cells are thought to express TCR-CD3 complex on the cell surface.

It is well known that TPA induces the differentiation and expression of cell surface antigens in various hematopoietic cell lines and in fresh leukemia cells. TPA stimulation of thymocytes, peripheral T cells and some T cell lines has been reported to induce the expression of IL-2 receptor on their cell surface membranes.[21-24] It was of interest to know whether TPA could induce the IL-2 receptor (Tac antigen) on pre–T-ALL cells that are the counterpart of normal immature precursor thymocytes. In the present study, all of four pre–T-ALL cells began to express Tac antigen after 12 hours of incubation with TPA. However, leukemia cells of three cases without rearrangement of TCR β and TCR γ genes expressed no new T cell antigen by the incubation with TPA for up to 72 hours. These findings suggest that the intracytoplasmic pathway for Tac antigen induction has already existed in immature pre–T-ALL cells expressing no TCR-CD3 complex on their cell surface membranes. TPA stimulation of the leukemia cells of case no. 4, which showed CD3 positive with both TCR β and TCR γ gene rearrangements, induced not only IL-2 receptor but also CD4 and CD8 antigens. It is unlikely that the contaminated PB-T cells in leukemia samples have proliferated by TPA stimulation, and CD4 and CD8 antigens have appeared on these normal cells, because PB-T cells were depleted from leukemia samples, and CD2 cell surface phenotype did not appear at all. Therefore, it is suggested that only late stage of precursor thymocytes with the expression of TCR-CD3 complex will have the capacity to differentiate into more mature stages of thymocytes accompanying the expression of CD4 and CD8 with TPA stimulation. The binding assay of ^{125}I-IL-2 to those pre–T-ALL cells revealed that IL-2 does bind to the IL-2 receptor on those cells with low affinity. Although the effect of IL-2 on early thymocytes is controversial, our data shows that exogeneous IL-2 does not influence the proliferation of TPA-stimulated pre–T-ALL cells.

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Localization of Surface vWF on Resting and Stimulated Platelets

By Jacob H. Rand, Ronald E. Gordon, Ronald R. Uson, and Barry J. Potter

We used immunoelectron microscopic localization techniques to investigate whether platelets stimulated by ADP or ristocetin in the plasma milieu bind von Willebrand factor (vWF) to their surfaces. We found by both peroxidase- and ferritin-based methods that unstimulated platelets lack vWF on their surfaces, whereas platelets that are stimulated with ADP or ristocetin have vWF associated with their surfaces. The specificity of the findings was confirmed by absorption studies using severe von Willebrand disease (vWD) and hemophilic plasmas. The anti-vWF antibodies were blocked by incubation with hemophilic plasma but not by incubation with severe vWD plasma. Thus, in the plasma environment, in the presence of fibrinogen, vWF becomes associated with the platelet surface subsequent to stimulation with ADP or ristocetin.

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VON WILLEBRAND FACTOR (vWF) is a multimeric glycoprotein that supports the adhesion of platelets to vascular subendothelium under high shear rate conditions.[1] vWF can bind to two different platelet binding sites, glycoprotein Ib (GP-Ib) and the glycoprotein IIb/IIIa complex (GP IIb/IIIa). Binding to GP-Ib requires the presence of ristocetin,[2-5] and binding to GP-IIb/IIIa requires stimulation by agonists such as ADP and thrombin.[6,7] Since a physiologic counterpart for ristocetin has not been clearly identified, and since it has been demonstrated that, in the normal plasma milieu, fibrinogen would block the ability of the GP-IIb/IIIa complex to bind vWF present in the external milieu,[8-10] we decided to investigate whether vWF binds to platelets stimulated by ristocetin and by ADP using immunoelectron microscopic localization techniques. We examined binding of vWF morphologically, under conditions that would minimize the potentially perturbing influences of platelet washing steps and vWF purification and labeling.

MATERIALS AND METHODS

Antisera. Monospecific rabbit anti-human vWF was prepared as previously described.[11] Horseradish peroxidase-conjugated sheep Fab anti-rabbit IgG (HRP-SARG) and ferritin-conjugated SARG were obtained from Polysciences (Philadelphia). Controls for specificity of antigen localization included absorption of anti-vWF in plasma from a patient with severe classical hemophilia-factor VIII deficiency (George King, Overlook, KS), which contained normal levels of vWF and lacked coagulation factor VIII, and absorption in plasma from a patient with severe recessive von Willebrand disease (vWD) (George King, Overlook, KS), which was deficient in coagulation factor VIII and lacked detectable levels of vWF. Rabbit anti-human thyroglobulin was obtained from Dakopatts (Dako, Santa Barbara, CA).

Platelets. Normal blood was collected by clean venipuncture into a final concentration of 0.11 mol/L sodium citrate. Platelet-rich plasma (PRP) was separated after centrifugation at 250 g for ten minutes at room temperature. Platelet counts were maintained at ~300,000/μL by dilution with PRP. Aliquots of PRP with volumes of 0.9 mL were then incubated at 37°C for 15 minutes with constant rotation following the addition of 0.1 mL of the following materials: ristocetin (Lenau, Denmark), at a final concentration of 1.5 mg/mL, ADP (Sigma, New York) at a final concentration of 20 μmol/L, and iso-osmolar phosphate buffered saline (PBS) pH 7.2. Under these conditions both ristocetin and ADP-stimulated platelets formed fine aggregates. Following the incubation periods, the PRPs were fixed by addition of an equal volume of 4% paraformaldehyde in PBS for 30 minutes. The platelets were then centrifuged for five minutes in an Eppendorf microfuge (Brinkman Instruments, Westbury, NY) and prepared for immunoelectron microscopy.

Immuno-localization. Following fixation, the platelets were resuspended and washed for 12 hours in PBS-10% sucrose. The washed platelets were then resuspended in a 1:40 dilution of rabbit anti-vWF, as previously described, in which they were incubated for one hour at 37°C. Again, the platelets were centrifuged and washed three times in PBS-10% sucrose. The platelets were then resuspended in 1:40 dilution of SARG (1.25 μg/mL) coupled to either HRP or ferritin (Polysciences, Phila., PA) and then incubated for one hour at 37°C. After three ten-minute washes in PBS-10% sucrose, as described above, the platelets were treated with 3% gluteraldehyde in PBS-10% sucrose in order to fix the antibodies in place. The platelets were then resuspended and washed three times in PBS. In the case of the ristocetin and ADP stimulated platelets, fine aggregates were observed during the washing steps. The platelets that had been treated with HRP-SARG were then incubated for seven to ten minutes in a 0.5% solution of 3.3-diaminobenzidine tetrahydrochloride (DAB) at a final concentration of 0.01% H_2O_2. Both the HRP-SARG and ferritin treated tissues were then treated with 1% unbuffered osmium tetroxide, were dehydrated with ethanol followed by propylene oxide, and were then embedded in Epon 812.

Controls. Several controls were used to check for the specificity of antigen localization. In order to ensure that we were not localizing nonantigenically bound antibody, platelets were also treated with nonimmune rabbit serum before they were treated with HRP-SARG and DAB. As noted above, in order to check for antibody specificity, the rabbit anti-vWF was absorbed in plasma from a patient with classical hemophilia-factor VIII deficiency and in a severe vWD plasma. Additional controls included treatment of platelets with SARG followed by DAB without any preceeding rabbit antiserum, and treatment of platelets with DAB alone. In order to check whether localization might be due to nonspecific trapping of protein from plasma during the course of processing, platelets activated by ADP and ristocetin were also incubated with rabbit anti-human thyroglobulin followed by staining with both the ferritin and the peroxidase methods.

One-μm thick sections were cut from all blocks and examined by light microscopy. The presence and distribution of dense precipitates were evaluated under double-blind conditions. Areas from each block were sectioned for transmission electron microscopy. The

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Table 1. Immunoelectron Microscope Localization of vWF on Platelet Membranes

	Unstimulated Platelets	ADP	Ristocetin
Rabbit anti-vWF	−	+	+
Rabbit anti-vWF absorbed in hemophilic plasma	−	−	−
Rabbit anti-vWF absorbed in vWD plasma	−	+	+
Nonimmune rabbit serum	−	−	−

sections were examined without additional staining with heavy metal salts.

RESULTS

Platelets treated with anti-vWF probed by ferritin-conjugated antibodies were more revealing than those probed with peroxidase conjugated antibodies. In each instance, platelets treated with ristocetin and ADP demonstrated significant binding of vWF between adjacent platelets (Table 1, Figs 1 and 2). Interestingly, there was a paucity of labeling at sites away from platelet adjoinment.

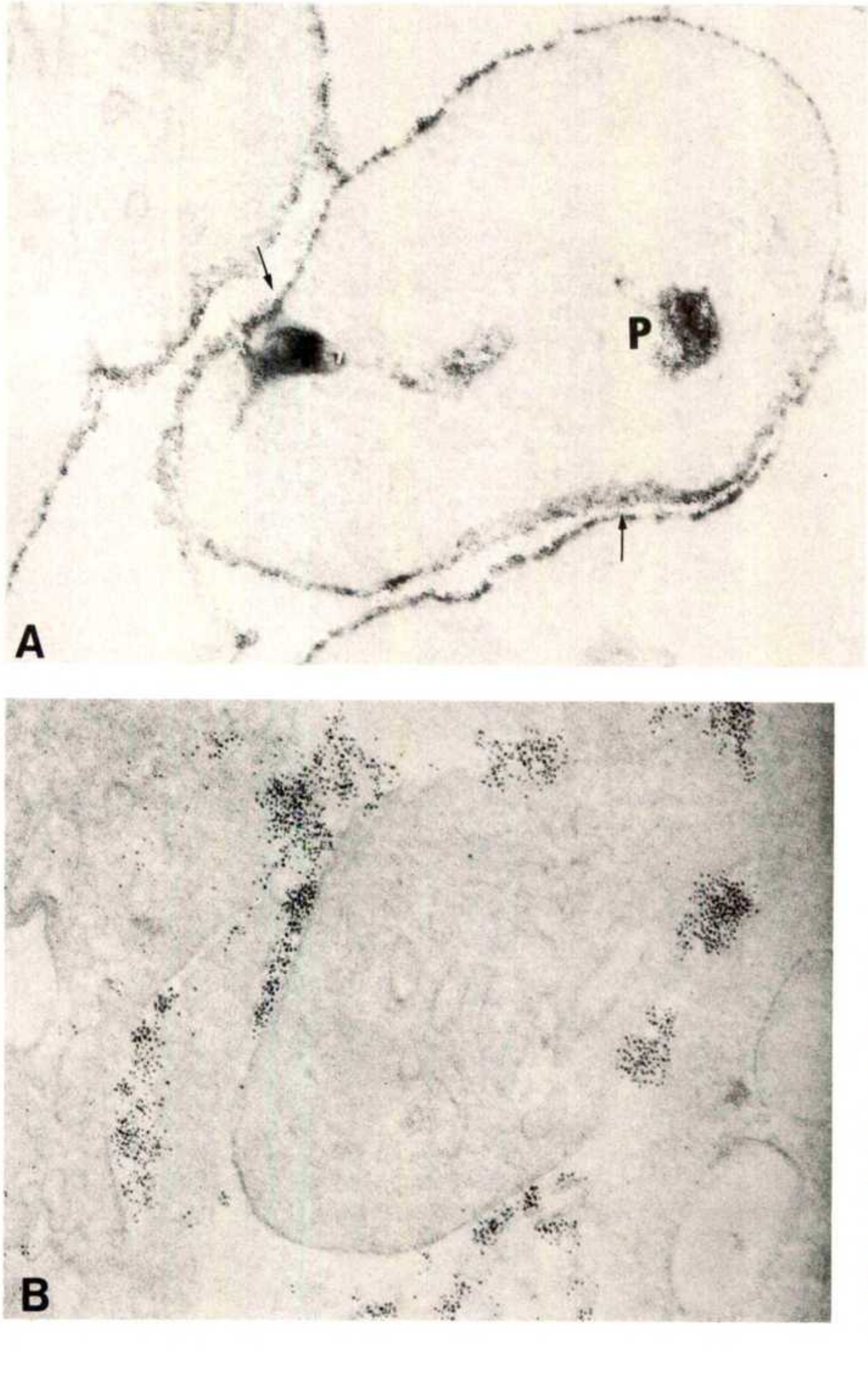

Fig 1. Electron micrographs of platelets treated with ristocetin, fixed in paraformaldehyde and sequentially treated with rabbit anti-human vWF, sheep anti-rabbit IgG coupled with (A) horseradish peroxidase and (B) ferritin, and then fixed with gluteraldehyde. The peroxidase-treated tissue was then incubated with 3,3-diaminobenzidine with H_2O_2 before embedment in Epon 812. Thin sections observed without prior staining with heavy metal salts exhibited significant vWF binding (arrows) to plasma membranes of adjoining platelets. Peroxidase labeled granules (P) are present within platelets. (A, Magnification ×56,000; B, magnification ×72,600.)

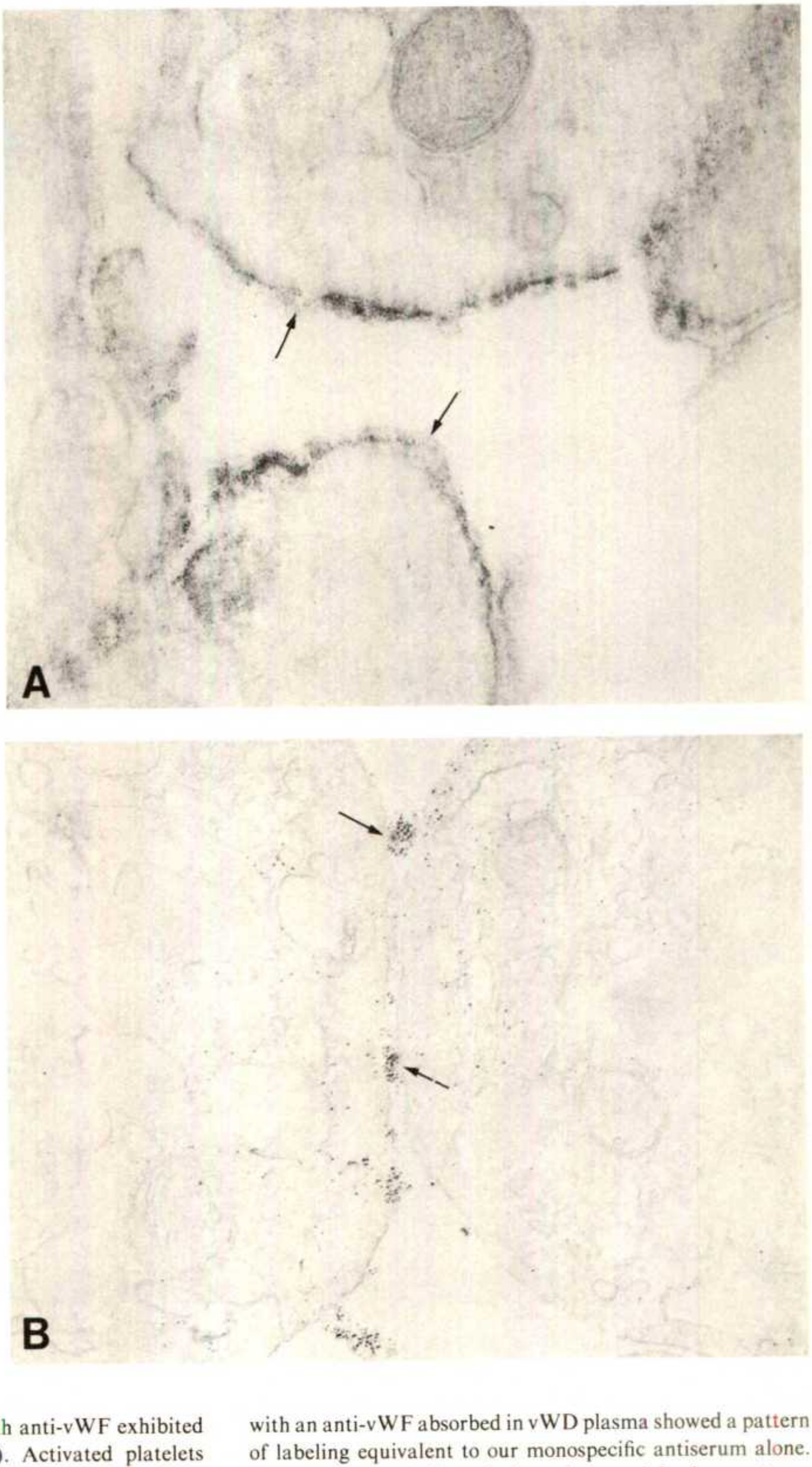

Fig 2. Electron micrographs of platelets treated with ADP and processed as described in Fig 1. The platelets exhibit sites of vWF binding (arrows) on the surfaces of adjacent platelets treated with antibody coupled to (A) horseradish peroxidase and (B) ferritin. (Magnification ×52,800.)

PBS-treated platelets incubated with anti-vWF exhibited little or no surface labeling (Fig 3). Activated platelets incubated with anti-vWF absorbed in hemophilic plasma followed by the second conjugated antibodies showed no surface labeling (Fig 4). Activated platelets treated with anti-thyroglobulin antibodies also showed no surface labeling. Platelets treated with SARG followed by DAB and by DAB alone, showed no surface labeling. Platelets incubated with an anti-vWF absorbed in vWD plasma showed a pattern of labeling equivalent to our monospecific antiserum alone. These controls indicated that the precipitation patterns observed represent specificity bound vWF and not trapped vWF, another antigen, or nonspecific immunoglobulins.

Since the platelets were not stained with heavy metal salts, ultrastructural elements such as platelet granules were not well defined. Sections treated by the peroxidase method with

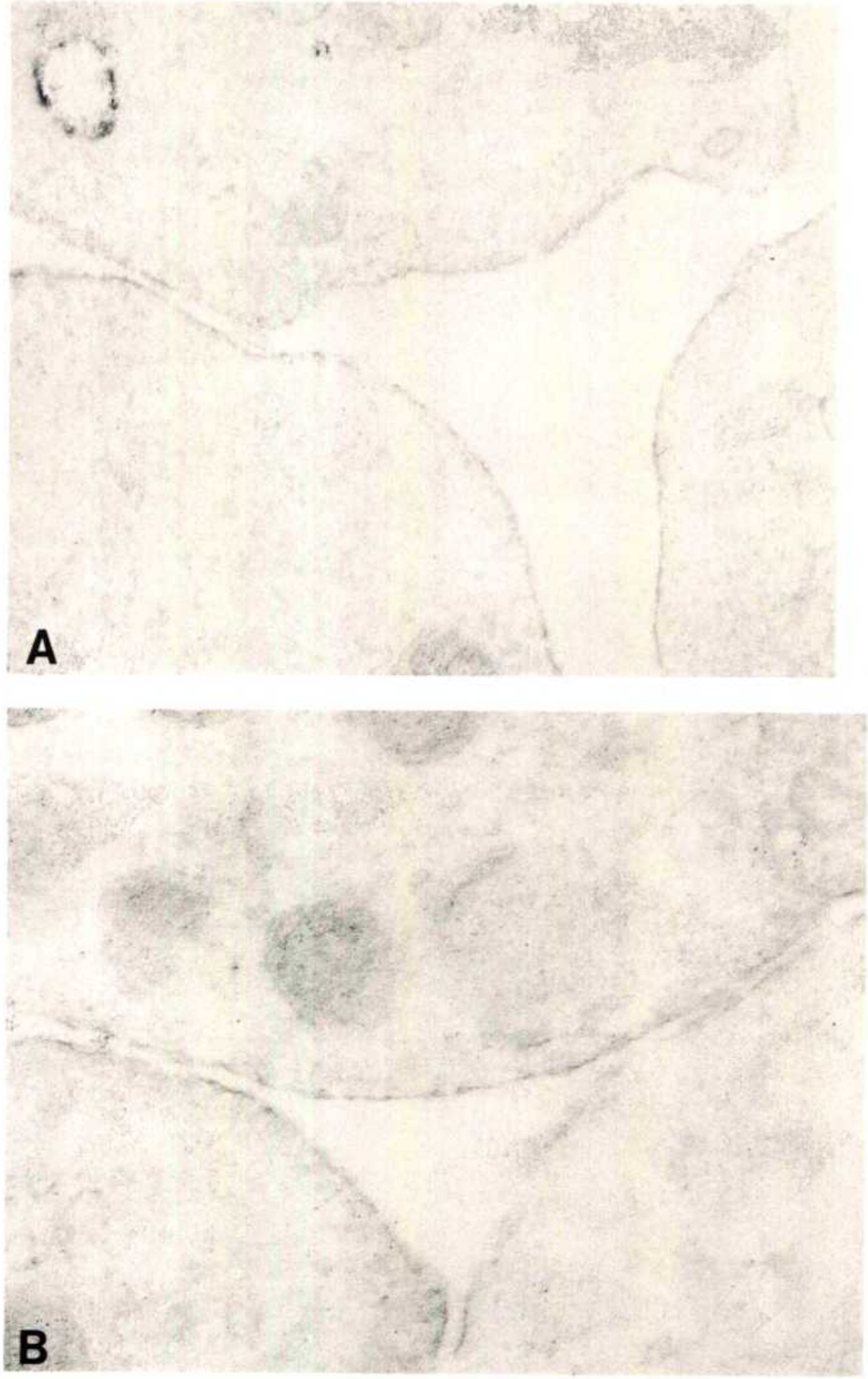

Fig 3. When PRP is incubated with PBS in place of ristocetin or ADP, fixed with paraformaldehyde and then processed as described in Fig 1, there are few or no sites of vWF localization detectable on the platelet surfaces as seen with (A) horseradish peroxidase and (B) ferritin coupled antibodies. (A, Magnification ×82,500; B, magnification ×59,400.)

DAB showed peroxidase labeling granules (Figs 1A and 3A). Because of endogenous peroxidase activity, antibody-linked peroxidase could not be distinguished from endogenous activity in the granules.

DISCUSSION

There have been several studies of vWF binding to platelets. These demonstrated ristocetin-dependent binding of vWF to binding sites on platelets by using I^{125}-vWF derived from lyophilized factor VIII concentrate or cryoprecipitate and washed platelets that had been collected in EDTA.[3-5] With respect to binding in the absence of ristocetin, Fujimoto et al demonstrated that thrombin as well as ADP and Ionophore A23187 induces a receptor for I^{125}-vWF in washed platelets collected from citrated blood.[6] Ruggeri et al showed that the ristocetin induced binding of vWF by platelets is mediated by GP-Ib, whereas the thrombin induced binding of vWF is mediated by GP-IIb/IIIa.[7] However, Pietu et al demonstrated that GP-IIb/IIIa does not bind vWF in the presence of normal fibrinogen levels.[8] Schullek et al, in a system using an I^{125}-tagged monoclonal anti-vWF as a probe, showed that thrombin and ADP/epinephrine induced bind-

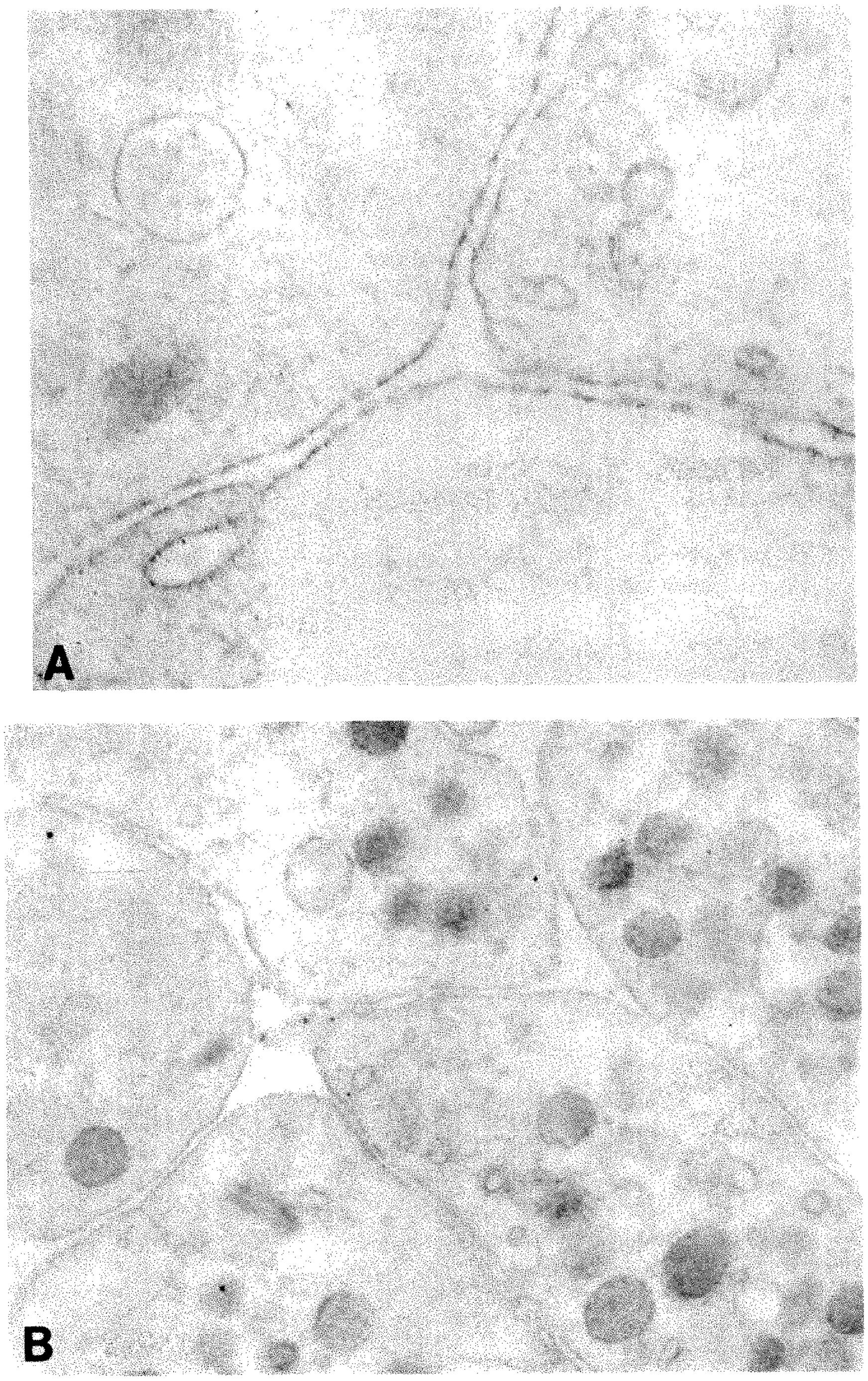

Fig 4. Micrographs of technique and immunologic controls all exhibit the absence of either (A) peroxidase reaction product or (B) ferritin as evidenced here by the lack of staining on platelets treated with ristocetin, fixed, and then incubated with antibodies to vWF which had been previously absorbed in hemophilic plasma. (A, Magnification ×66,000; B, magnification ×28,000.)

ing of vWF to washed platelets does not occur in the presence of plasma.[10]

The evaluation of vWF binding to platelets is frought with difficulties. The purified vWF may contain degraded subunit forms even under relatively optimal conditions.[11] The ligand is heterogeneous, consisting of a range of multimer sizes.[12,13] There is ambiguity about the nature of the platelet binding site with GP-Ib binding vWF only in the presence of ristocetin, and GP-IIb/IIIa, although capable of binding vWF in purified systems, appearing to function primarily as a fibrinogen binding site in plasma. The isolation and washing of platelets may perturb surface binding sites. Finally, vWF present within the platelet's α-granule[14] may interfere with tracers by binding to surface sites during either isolation and washing of platelets, or during platelet stimulation. In considering the complexity of the problem, we chose to use immunolocalization techniques that would perturb the system as minimally as possible.

In this study, we found that both ristocetin and ADP promote clusters of precipitate representing vWF that are most prominent at platelet contact points with other platelets. Thus, in addition to vWF's role in promoting platelet adhesion to vascular subendothelium, we observe morphologic evidence for the presence of vWF at platelet contact sites. Our studies do not relate the surface vWF to the putative receptors, GP-Ib or GP-IIb/IIIa.

The specificity of the observed localization patterns is supported by the equivalent findings with antiserum that was absorbed in vWD plasma. The lack of observable precipitates with anti-vWF that had been absorbed in factor VIII deficient plasma indicates that the precipitates did not reflect localization of fibrinogen, coagulation factor VIII, Fc receptor binding of immunoglobulins or, in the case of HRP-SARG, endogenous peroxidase activity. The latter two possibilities have also been excluded by the negative results of our additional controls which included: (1) nonimmune rabbit serum, (2) the substitution of a rabbit antibody against human thyroglobulin, (3) the omission of a primary rabbit anti-serum, and (4) treatment beginning with the peroxidase substrate, DAB.

The localization of vWF between activated platelets may relate to its role in supporting the formation of platelet thrombi. vWF is an adhesive glycoprotein that supports platelet adhesion to exposed vascular subendothelium[1] and also appears to support platelet thrombus formation.[15] In the case of platelet adhesion, vWF is present on the subendothelial surface where it promotes adhesion.[16-18] However, in order for vWF to play a similar role in platelet thrombus formation, vWF from plasma, or released from endogenous stores must attach to platelet surface binding sites. Evidence for such binding is presented in this study.

The source(s) of the vWF that associates with the platelet surface following stimulation by ADP in the plasma environment is not yet known. As noted above, it is clear that washed platelets stimulated by ADP can bind exogenous vWF when fibrinogen is absent. In the case of unwashed platelets stimulated in plasma, the surface bound vWF might be released or externalized α-granules, might yet be bound from plasma, or might come from both sources.

In conclusion, our immunolocalization studies have demonstrated that following activation, vWF shows distinct localization patterns on the platelet surface. This association occurs even in the plasma milieu, in the presence of normal fibrinogen levels. This surface vWF may explain the observation that vWF promotes the presence of platelet aggregates on vascular subendothelium.[15]

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Specific Adsorption of HTLV-I to Various Target Human and Animal Cells

By Karen Krichbaum-Stenger, Bernard J. Poiesz, Paul Keller, Garth Ehrlich, Jerrie Gavalchin,
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In this report, we describe a flow cytometric analysis of HTLV-I specific binding to fresh and cultured cells on a single cell basis. This assay uses rhodamine hydrocarbon tagged, purified HTLV-I virions according to the procedure originally described for avian retroviruses. Successful HTLV-I transmission was detected by analysis of integrated HTLV-I DNA, virion-associated reverse transcriptase, and/or intracellular HTLV-I core antigen p19 expression. Only a specific virus–cell interaction was detected because nonrhodamine-tagged homologous virus or related HTLV-II interfered with tagged HTLV-I binding. In contrast, an unrelated, nonlabeled animal retrovirus was unable to block tagged HTLV binding. Of the cell lines tested, 2 nonlymphoid mammalian and 3 human lymphoid bound significantly high to moderate levels of HTLV-I–tagged virions. The other three human lymphocyte cell lines were insensitive to HTLV-I adsorption. A direct correlation was observed between HTLV-I binding sites and infectivity of human lymphoid cells alone and not other nonlymphoid animal cells. Fresh normal human mononuclear cells bound low levels of HTLV-I virions. As expected, T lymphocytes demonstrated more binding than did the non-T cell population. Enhancement of HTLV-I cell binding in a subpopulation of mononuclear target cells was achieved with phytohemagglutinin (PHA) activation and interleukin 2 (IL2) stimulation, which correlates well with previously published infectivity studies.
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THE HUMAN T CELL lymphoma/leukemia virus I and II are associated with adult T cell leukemia (ATL)[1-5] and T cell hairy cell leukemia, respectively.[6] Functionally, both viruses transform T lympocytes in vitro. In vitro infection of a wide range of human and animal cells by both viruses has been reported. Using cocultivation techniques, normal, T$_4$, T$_8$, and non-B/non-T lymphocytes can be infected.[7-10] Lymphocytes from several other species can also be infected.[11] Human-derived cell lines, including B lymphocytes, osteogenic sarcoma cells, fibrosarcoma cells, diploid fibroblasts, and endothelial cells can also be infected.[12-16]

The only nonhuman, nonlymphoid cell line capable of HTLV-I transmission is feline kidney fibroblasts.[15] The in vitro host range of HTLV-II differs slightly from HTLV-I. Although equally transmissible to human T lymphocytes, HTLV-II more efficiently infects B lymphocytes and is not transmitted to human fibrosarcoma, human osteosarcoma, or feline kidney fibroblasts.[15]

Despite their varied in vitro tropism, however, HTLV-I and HTLV-II have been associated only with human malignancies exhibiting the T$_4$ T lymphocyte cell surface phenotype.[3,6] This in vivo cellular tropism of HTLV-I may be regulated at several points in the virus life cycle: (a) viral adsorption and penetration, governed by specific viral target membrane receptors; (b) viral genomic integration into target cell DNA; and (c) RNA transcription of proviral DNA, requiring noncoding, viral-specific, regulatory sequences called long terminal repeats (LTRs).

Several lines of evidence support the concept that the cell surface receptors may be a key in the determination of HTLV-I and HTLV-II cell tropism in vivo. First, although the major envelope glycoproteins of HTLV-I and HTLV-II are distinct, they are more homologous than the LTR transcriptional regulatory sequences of HTLV-I and HTLV-II.[17,18] In addition, by vesicular stomatitis virus (VSV) pseudotype interference studies, these two related human retroviruses appeared to share the same cell membrane receptors.[15] Another argument involves the lack of site-specific integration of HTLV-I into cellular chromosomal DNA.[19,20] Examination of HTLV-I proviral DNA integration sites in fresh and cultured cells has indicated that the virus does not integrate in any preferential site or chromosome.[19,20] Thus, this virus does not appear to directly regulate RNA transcription of an adjacent cellular gene by *cis* activation. Last, recent data suggest that the molecular mechanisms of HTLV-I transformation involves *trans* activation of HTLV-I expression itself and possibly a yet unknown, oncogenic gene at a distant chromosomal location by the HTLV-I pX protein, LOR.[21] These observations suggest that HTLV-induced cell transformation is primarily dependent on the susceptibility to virus attack at the specific cell membrane receptor level. Accordingly, the present studies focus on the development of an assay for relative quantitation of HTLV-I and HTLV-II cell membrane binding sites by flow cytometric analysis of rhodamine hydrocarbon-labeled HTLV bound to fresh and cultured cells on a cell–cell basis, as previously described for avian retroviruses.[22-24]

MATERIALS AND METHODS

Cells. The following cell lines were maintained in culture: HUT 102-B2, an HTLV-I–producing, mature neoplastic T lymphocyte from a lymph node of an ATL patient[3]; MT-2, umbilical cord blood mature T lymphocyte exogenously infected with HTLV-I[25]; Mo-T, HTLV-II–producing, human T lymphocyte from F. Ruscetti, National Institutes of Health (NIH); and the virus negative lines;

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Molt-4, an immature T lymphocyte derived from a patient with acute lymphocytic leukemia; HUT 78, mature T lymphocytes derived from a patient with cutaneous T cell lymphoma[26]; FR-45, continuous human B lymphocytes from a normal donor from F. Ruscetti, NIH; NK-41, continuous human natural killer cells from a normal donor established by F. Ruscetti, NIH; VERO, an African green monkey kidney cells; and SC-1, a wild mouse fibroblasts from C. Kozak, NIH. Normal adult peripheral blood mononuclear cells were obtained by centrifugation of heparinized blood on a Ficoll-Hypaque density gradient. Activated T cells were generated by culturing the above cells in RPMI 1640 media, 10% fetal calf serum (FCS), 5 μg/mL phytohemagglutinin (PHA), and 10% delectinated, partially purified human interleukin 2 (IL 2) (Cellular Products, Buffalo).

T cell and non-T cell–enriched population were prepared by forming E rosettes with treated sheep RBCs (SRBCs) and performing sedimentation on lymphocyte separation media (Litton Bionetics, Kensington, MD).[27]

Purification of retrovirus particles. Virions of HTLV-I, HTLV-II, or a B tropic, ecotropic murine leukemia virus (2557) from a BALB/c mouse were purified from media conditioned by the following cell lines; HUT 102-B2, Mo-T, and SC-1, respectively. To prevent possible disruption of intact whole virions or removal of their envelope proteins, a gentler purification was performed in comparison to previously described more stringent virus purification techniques.[3] After removal of cultured cells from 2 to 20 L growth media (RPMI 1640, 10% FCS, 1% penicillin-streptomycin by sedimentation at 870 g for 15 minutes, virions were concentrated by centrifugation of the supernatant at 30,500 g for 45 minutes and resuspended in 10 mmol/L Tris-HCl, pH 7.4, 0.1 mol/L NACl, 1 mmol/L EDTA (TNE buffer). Pooled virions were then spun through 30% sucrose in TNE buffer onto a 45% sucrose cushion by ultracentrifugation on two consecutive discontinuous 30% to 45% sucrose density gradients at 89,400 g for 2.5 hours. Virions were stored in 200-μL aliquots at −70°C.

Reverse transcriptase assay. Virus particles were quantitated according to the amount of reverse transcriptase activity present per lot of purified virions. Assessment of reverse transcriptase activity was carried out as previously described.[4] Results were expressed in values of pmoles of ^{3}H-deoxyguanidine monophosphate incorporated per hour.

Labeling of virus with a fluorescent membrane probe. Rhodamine-16 (R-16) is a fluorescent dye (rhodamine) covalently linked to a saturated hydrocarhon 16 carbons long.[22] The synthesis of similar membrane probes is described in ref. 23. The hydrocarbon chain causes the fluorescent dye to partition into lipid-rich viral envelopes and to remain there preferentially.[23] The labeling technique that maintains infectivity of the virus and target cell viability was established previously.[22] In brief, virions were conjugated with rhodamine-16 (HTLV/R-16) by incubating gradient purified virus in a final concentration of 0.25 mmol/L R-16 in 5% dimethyl sulfoxide. Unbound R-16 and any free R-16 were removed by two consecutive discontinuous sucrose velocity gradient centrifugations. The virus was concentrated on a glycerol cushion, and the buffer above the cushion was analyzed for the presence of any remaining free R-16.

Sample preparation for flow cytometric analysis. The adsorption procedure of R-16–labeled virions to various cells was performed as follows: Various concentrations (1 HTLV-I/R16 concentration refers to the equivalent number of virions containing 50 pmoles of reverse transcriptase activity) of R-16–labeled virions in glycerol and TNE buffer were incubated with 7.5 × 10^5 target cells in suspension in 0.2 mL minimal essential medium (MEM), 1% FCS and 2 μg/mL polybrene at 4°C, or room temperature. At 4°C, adsorption of HTLV-I occurs without subsequent penetration, whereas both viral adsorption and penetration of the target cell are possible at 25°C.[28] After the virus–cell interaction, cells were spun at 400 g for 5 minutes at 4°C, supernatant was removed, and cells were resuspended in 0.4 mL fresh medium (MEM, 1% FCS, and polybrene). Samples were filtered through 44-μm nylon mesh to remove clumps and were kept on ice during flow cytometric analysis to maintain cell viability and to prevent significant viral penetration.[22]

Multiparameter flow cytometric analysis. Flow cytometric (FCM) measurements were performed on the Coulter EPICS V (Coulter Electronics, Hialeah, FL) equipped with a 4-W argon laser. Fcm measurements (relative fluorescent intensities) of rhodamine-labeled HTLV-I adsorbed cells resulted from excitation by the 514-nm line of the cytometer's argon-ion laser, and the fluorescence emitted was monitored by detectors at wavelengths >610 nm using 530-nm short-pass interference and 570-nm long-pass absorbance filters.[22] Data were expressed in histogram form. Each histogram is based on 10,000 events fitting the forward angle and 90° light-scatter gates to reduce the contribution of dead or clumped cells; the rhodamine fluorescence was quantitated. Each sample was screened for background fluorescence representing autofluorescence of cells in the absence of rhodamine. The distribution of fluorescence-positive cells was expressed on the *y* axis and the increasing intensity of fluorescence was expressed on the *x* axis on a log scale. The mean channel fluorescence data for all samples were converted to relative linear fluorescence values using a log to linear conversion method[29] to allow better assessment of experiments.

Competitive inhibition studies using HTLV. Binding studies for the competition analysis were carried out as described above, except that prior to the addition of rhodamine-tagged virions, cells were incubated for 1½ to 2 hours with varying amounts of unlabeled HTLV-I, HTLV-II, and/or murine leukemia virus (MLV) (as determined by relative reverse transcriptase activities).

HTLV transmission assay. Cocultivation of HTLV-I–positive donor, neoplastic T cells with various human lymphoid target cells was carried out as described.[8] Resultant cell lines were assessed for HTLV-I production by assaying for extracellular reverse transcriptase and intracellular HTLV-I p19 core protein. The presence of integrated HTLV-I proviral DNA in the transformed cells was determined by dot blot or Southern blot hybridization using an HTLV-I cloned DNA probe.[8]

HTLV p19 detection. Indirect immunofluorescent assays for HTLV-I p19 using an anti–HTLV-I p19 monoclonal antibody were performed by a previously described technique.[8]

Nucleic acid hybridization. High-mol-wt DNA was extracted from the target, donor, and cocultivated cells and transferred onto nitrocellulose filters as previously described.[8] Dot blot or Southern blot hybridization analysis was performed with these nitrocellulose filters and a radiolabeled HTLV-I *gag-pol* DNA sequence, pATK 32 (a gift of Dr M. Yoshida).

RESULTS

Binding site saturation. Figure 1 shows an HTLV-I/R-16 MT-2 cell binding saturation curve, established with increasing concentrations of gently purified HTLV-I labeled with R-16 and a constant number of target cells and time of adsorption. The observed relative mean fluorescence increased in a linear manner at the lower concentrations of HTLV-I/R-16. The plateau of virus adsorption was reached at an HTLV-I/R-16 inoculum containing 25 pmoles of reverse transcriptase, indicating significant saturation of binding sites. For this reason, the concentration of HTLV-I/R-16 chosen to bind 7.5 × 10^5 target cells in a standard

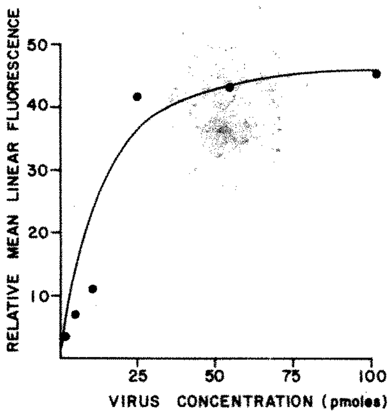

Fig 1. A saturation curve of HTLV-I binding to MT-2 cells using R-16–labeled HTLV-I and flow cytometry; 7.5 × 10^5 MT-2 cells were adsorbed for 30 minutes with increasing concentrations of HTLV-I/R-16 and analyzed in an EPICS V flow cytometer as described in the Materials and Methods section. The *x* axis scale represents the number of pmoles of reverse transcriptase activity in each virus adsorption sample.

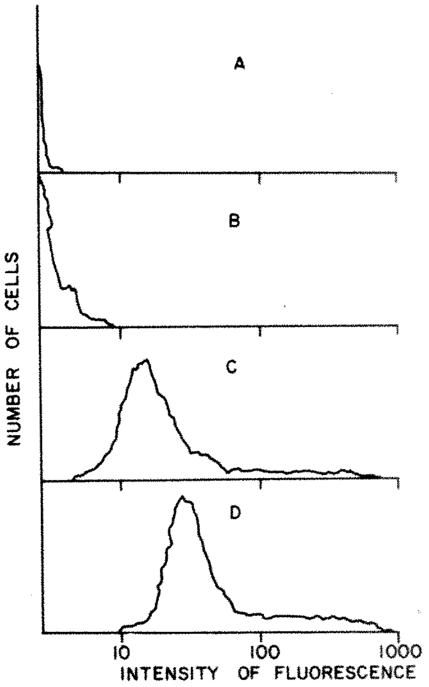

Fig 2. The distribution of HTLV-I binding sites on HTLV-I–susceptible HUT 102-B2 cells. The frequency of cells in each fluorescence category is plotted on the *y* axis against the cytometer detected intensity of fluorescence on the *x* axis (scale 0 to 1,000). (A) Control, unadsorbed HUT 102-B2 cells.[1] (B) HUT 102-B2 cells incubated with virion-free supernatants from an equivalent amount of HTLV-I/R-16 virus preparation as in D after removal of the labeled virions by centrifugation.[2] (C) Adsorption of an equivalent amount of HTLV-I/R-16 as in D to HUT 102-B2 binding sites after competition with unlabeled HTLV-I.[15] (D) HTLV-I/R-16 bound to HUT 102-B2 culture cells.[30] In Figs 2–6, numbers in parentheses indicate relative mean fluorescence.

assay was 50 pmoles of reverse transcriptase containing HTLV-I/R-16.

HTLV-I cell membrane binding assay. In all experiments, viable and nonviable cells were simultaneously analyzed by using forward-angle and 90° light-scatter parameters, but only the R-2 signals from 10,000 viable cells were measured. Figure 2 illustrates characterisic binding curves of free R-16 and R-16–labeled HTLV-I binding, respectively, to the prototype HTLV-I–producing cell line HUT 102-B2. For each cell population examined, the mean fluorescence bound per cell was recorded and the autofluorescence (fluorescence of cells in the absence of rhodinated virus) was checked. The HUT 102-B2–bound HTLV-I/R-16 histogram (Fig 2D) demonstrates significant adsorption of R-16–labeled virions to HUT 102-B2 cells with the maximum number of cells being represented by a mean fluorescence of 30, whereas the background autofluorescence of the same cells were minuscule (Fig 2A). The amount of fluorescence diminishes twofold (mean fluorescence 15) when target cells are incubated with unlabeled HTLV-I prior to the addition of rhodamine-tagged virions (Fig 2C). To rule out binding of residual free R-16 to HUT 102-B2 cells, HTLV-I/R-16 virus preparations were centrifuged to remove the labeled virions, and the supernatant (free of virions) was then reacted with HUT 102-B2 cells (Fig 2B). Results indicate that the HTLV-I/R-16 preparations are minimally contaminated with unbound R-16 or free rhodamine (Fig 2B).

MT-2 target cells infected with HTLV-I were chosen for reproducibility studies. Multiple tests on the same sample of HTLV-I/R-16 adsorbed MT-2 cells (instrument variability) and between replicate samples of HTLV-I/R16–bound MT-2 cells (assay variability) at near saturation showed a high degree of reproducibility. The SEM was 1.35% for instrument variability and 2.65% for assay variability (data not shown).

Homologous HTLV competitive binding. Figure 3 shows the histograms of relative HTLV-I/R-16 adsorption to several target cells and the effects of homologous virus competition. HTLV-I/R-16 adsorption specificity was clearly indicated in 57% and 52% of viable HUT 102-B2 and VERO cells, respectively (shaded areas). In contrast, only 28% of the normal adult peripheral blood mononuclear cells (PBMs) with PHA activation and IL 2 scored positive for HTLV-I/R-16 binding sites. These percentages are minimal numbers representing the number of HTLV-I/R-16–bound cells with a fluorescent intensity greater than the fluorescent intensity of tagged virion-bound cells that competed with homologous virus. The VERO cells expressed the highest number of specific fluorescent binding sites, more than twofold and fourfold higher than HUT 102-B2 and normal nonactivated adult PBMs, respectively (Fig 3B). Likewise, HUT 102-B2 cells showed an almost twofold greater rhodamine fluorescence over the PBMs tested (Fig 3A). The level of fluorescent intensity correlates with the surface area of these cells (data not shown). Homologous HTLV-I binding competition, with equal inoculums of unlabeled and labeled HTLV-I, reduced membrane bound fluorescence twofold in both the VERO and HUT 102-B2 cells. A concentration of unlabeled HTLV-I tenfold greater than HTLV-I/R-16 almost completely inhibited binding to HUT 102-B2 cells (data not shown). The activated adult PBM

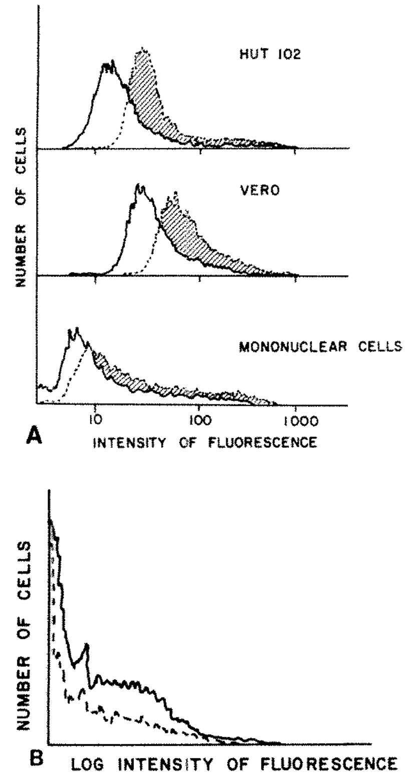

Fig 3. (A) HTLV-I adsorption to various target cells. The African green monkey kidney cell line, VERO, demonstrates the highest degree of rhodamine fluorescence, followed by HUT 102-B2 cells, and finally normal adult activated PBMs. The solid-line histogram represents cellular fluorescence resulting from the homologous competition between HTLV-I/R-16 and unlabeled HTLV-I virions in HUT 102 (21) VERO (41), and mononuclear cells (13), whereas the broken-line histogram represents the distribution of cellular binding sites of HTLV-I/R-16 alone without competition in HUT 102 (41), VERO (81), and mononuclear cells (20). Numbers in parentheses represent relative mean fluorescence. (B) HTLV-I adsorption to T-enriched (solid line) and non-T–enriched (broken line) PHA-activated normal PBMs.

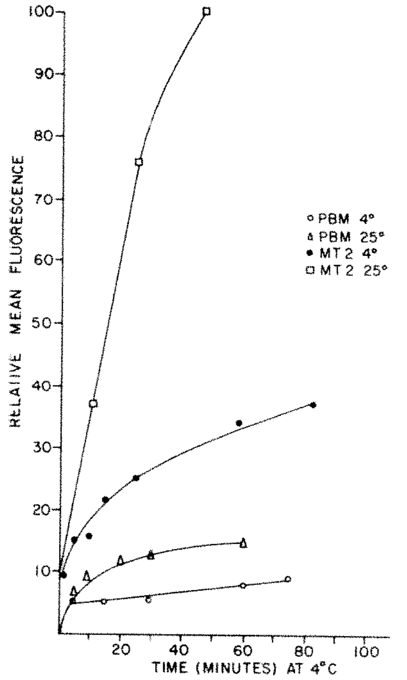

Fig 4. Effect of temperature on the binding rate of rhodamine-labeled HTLV-I virions to MT-2 culture cells and nonactivated adult PBMs. A constant amount of rhodamine-tagged HTLV-I virions were added to 7.5 × 10^5 target cells and incubated at either 4°C or 25°C for the time indicated on the *x* axis: MT-2 cells adsorbed with HTLV-I/R-16 at 25°C (–); MT-2 cells adsorbed with HTLV-I/R-16 at 4°C (O–O); PBMs adsorbed with HTLV-I/R-16 at 25°C (–); PBMs adsorbed with HTLV-I/R-16 at 4°C (O–O).

cells were more resistant to competition with unlabeled homologous HTLV-I (Fig 3C).

Kinetics of HTLV-I specific binding at 4°C and 25°C. To establish the rate of specific binding of HTLV-I/R-16 to susceptible MT-2 cells and fairly resistant adult fresh PBMs, the parameters of temperature and time were varied (Fig 4). At 4°C, the assay measures viral adsorption alone, whereas at 25°C the virus can bind and penetrate the cells, possibly allowing for multiple sequential binding of a single cellular binding site.[28] Fluorescent signals were recorded at minute intervals from the initial interaction of HTLV-I/R-16 and target cells. The rate of binding of HTLV-I/R-16 to MT-2 cells at 25°C proved very rapid and linear. No plateau was observed for 120 minutes. At 4°C, the rate of binding of HTLV/I/R-16 to MT-2 cells was much slower; within 15 minutes, 50% of the maximum cell-bound fluorescence was attained (Fig 4). The rate of binding subsequently plateaued at 60 minutes, followed by only minimal additional binding. These results are in accord with well established kinetics of specific virus–cell membrane receptor interactions at 4°C.[28] In comparison, the maximum binding of HTLV-I/R-16 to fresh, nonactivated adult PBMs at 4°C and 25°C was much less. The adsorption plateau was reached at both temperatures more quickly than in MT-2 (Fig 4). As expected, the maximum bound PBM fluorescent was significantly higher at 25°C than at 4°C.

Assay sensitivity. The low limit of detectability of HTLV-I cell binding sites was determined by mixing a small proportion of known high HTLV-I binding site cells (MT-2) with a large proportion of cells with low HTLV-I cell binding activity (nonactivated PBMs). A mixture of 90% of PBMs and 10% MT-2 cells (Fig 5B) and another of 95% PBMs and 5% MT-2 cells (Fig 5C) showed 26% and 19% viable fluorescent cells above an intensity of 34 (the left edge of the MT-2 curve) whereas only 13% of the viable pure population of PBMs bound fluorescence above an intensity of 34 (Fig 5A). After subtraction of fluorescent-bound PBMs, the number of HTLV-I/R-16–bound MT-2 cells in these mixed populations were calculated as 13% and 16%, respectively. These results indicate that a subset of the PBMs was probably HTLV-I binding site positive and that as little as 5% and 10% HTLV-I cell membrane binding site-positive cells was accurately detected in these heterologous population of cells.

Binding competition with an unrelated retrovirus. To demonstrate further the specificity of this virus membrane binding assay, an ecotropic MLV, which has no significant

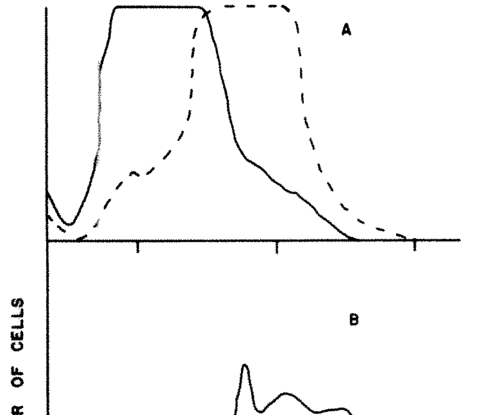

Fig 5. Sensitivity of the virus–cell binding assay. Histograms represent the frequency distribution of: (A) HTLV-I/R-16 bound to 100% PBM cells (O——O), HTLV-I/R-16 bound to 100% MT-2 cells (O---O). (B) HTLV-I/R-16 bound to a mixture of 90% PBMs and 10% MT-2 cells minus the histogram of HLTV-I/R-16 bound to 100% PBMs in A. (C) HTLV-I/R-16 bound to a mixture of 95% PBMs and 5% MT-2 cells minus the histogram of HTLV-I/R-16 bound to 100% PBMs in A.

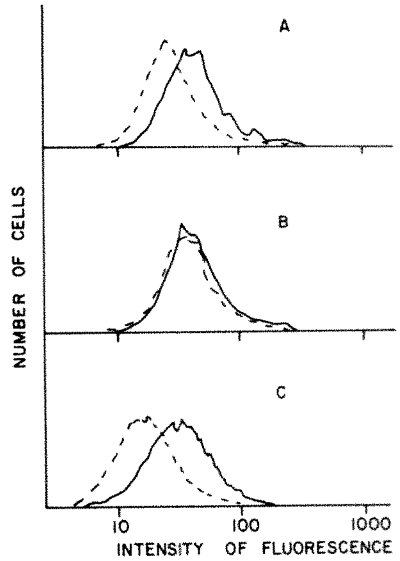

Fig 6. HTLV-I/R-16 binding competition with an unrelated retrovirus, MLV. Solid-line histograms represent frequency distribution of HTLV-I/R-16–bound MT-2 cells (43) in A and B, or MLV/R-16 bound SC-1 cells (31) in C without competition. Dashed-line histograms show distribution of fluorescent MT-2 cells reacted with homologous virus (HTLV-I) (29) in panel A or heterologous virus (MLV) (42) in B followed by adsorption of HTLV-I/R-16. In C, the dashed line represents frequency distribution of fluorescent-positive SC-1 cells exposed to homologous MLV prior to MLV/R-16 adsorption (16). Numbers in parentheses represent relative mean fluorescence.

primary nucleotide sequence homology to HTLV-I and a restricted mouse or rat cell host range was used in cross-interference studies.[30,31] Figure 6A shows histograms of MT-2 cells that revealed a twofold decrease in fluorescent intensity with HTLV-I homologous interference whereas only slight interference was visualized with MLV adsorption prior to HTLV-I challenge (Fig 6B). In contrast, MLV membrane binding to susceptible SC-1 cells and significant binding inhibition (fourfold) with homologous virus was clearly observed (Fig 6C).

Correlation between HTLV-I/R-16 adsorption and infectivity of target cells. Table 1 is a summary of HTLV-I/R-16 adsorption and attempted infection of human lymphoid target cells by cocultivation with HTLV–I producing donor cells. Table 1 is arranged in rank order of the highest to the lowest levels of HTLV-I/R-16 cellular adsorption. The data correlates directly with the transmission of HTLV-I to target cells by cocultivation with an equal number of irradiated, virus-positive T lymphoblasts. The MT-2, HUT 102-B2, MOLT 4, and normal adult-activated cycling PBMs bound HTLV-I/R-16 at mean fluorescence >10. The high relative mean fluorescence of HTLV-I/R-16 bound to HUT 102-B2 cells, is consistent with the high level of constitutive, endogenous infectious HTLV-I expression. Consequently, the amount of intracellular HTLV-I core antigen p19 detected by indirect immune fluorescent assay was equally elevated. Examination of chromosomal HTLV-I DNA by dot blot or Southern blot hybridization revealed intense dark dots or bands, indicative of multiple integrated copies of HTLV-I. In addition, virion-associated reverse transcriptase was detected with a specific template primer poly [rC]. • oligo [dG] in the presence of Mg^{2+}. MOLT 4, an immature T lymphoblast cell line, and normal activated adult mononuclear cells can be infected with HTLV-I, as indicated by results of similar virus transmission assays.[8] The adsorption of HTLV-I/R-16 to HUT 78, FR45, and NK41, is approximately sixfold less than that to HUT 102-B2 cells. Homologous untagged HTLV-I failed to interfere significantly with HTLV-I/R-16 bindings in this former cell group. These data suggested relatively fewer binding sites and a lack of affinity for HTLV-I binding sites, which correlates well with the lack of HTLV-I transmission.

Two nonlymphoid, nonhuman fibroblast cell lines tested in the same experiment with the human lymphoid cell lines were African green monkey kidney epithelioid cells (VERO) and wild mouse fibroblasts (SC-1). Both these cell types are large in comparison to fresh mature lymphoid cells. In agreement with this size correlation, SC-1 and VERO cells both had large numbers of HTLV-I/R-16 binding sites with relative mean fluorescence of 120 and 59, respectively (data not shown), which was reduced approximately twofold with an equal concentration of competitive homologous unlabeled HTLV-I. At present, no evidence of HTLV-I infectivity has

Table 1. Relationship Between HTLV-I Virus Adsorption and Infection in Human Lymphoid Cells

Target Cells	HTLV-I-Adsorption Relative Mean Fluorescence	HTLV-I Adsorption After Inhibition With Unlabeled HTLV-I (Relative Mean Fluorescence)	HTLV-I Infection: Reverse Transcriptase pmoles/hr/100 λ reaction Mixture	HTLV-I Infection: HTLV-I DNA	HTLV-I Infection: Immunofluorescence Cells Positive For HTLV-I p19 (%)
MT-2	35	15	10.50	+	99
HUT 102-B2	26	13	2.05	+	98
MOLT 4	17	10	1.79	+	NT
PMB+*	13	8.0	0.85	+	93
HUT 78	8.2	8.0	0.12	−	0
FR-45	8.0	8.0	0	−	0
NK-41	7.2	7.1	NT	−	0

NT, not tested.

*Activated normal adult peripheral blood mononuclear cells from normal donors.

been observed, but to prove conclusively a lack of HTLV-I transmission cocultivated target cells should be screened in a more sensitive assay such as the polymerase chain reaction coupled with Southern blot hybridization. The adsorption of virus to cell membranes does not always imply that the cell is permissive for infection. Susceptibility of cells to infection can be controlled at later points in the virus growth cycle beyond virus penetration into the cell.

HTLV-I adsorption to normal human mononuclear targets. Four normal donors were screened for HTLV-I/R-16 binding sites on their Ficoll-Hypaque density-gradient–purified mononuclear cells before and after activation of their T lymphocytes with PHA and growth in partially purified, delectinated IL 2 (Table 2). All nonactivated mononuclear target cells bound HTLV-I/R-16 to form a single peak of fluorescent cells. In contrast, the activated target cells were separated in two peaks of fluorescent cells based on intensity. The nonactivated PBMs were significantly more resistant to competition with unlabeled homologous HTLV-I, suggesting a lack of specificity. In three of four donors, one activated peak was significantly higher and the other lower than the nonactivated fluorescent cells (Table 2). In donor 4, the HTLV-I/R-16–bound cells with the highest intensity of fluorescence after activation were not significantly higher than the single peak of nonactivated target cells. These results were in accord with earlier studies showing that HTLV-I infection of normal mononuclear cells is more efficient in cycling, activated cells.[8] Analysis of T-enriched *v* non-T–enriched populations from these mononuclear preparations indicates that HTLV-I adsorption is greatest in the former population (Fig 3B).

Table 2. HTLV-I/R-16 Adsorption to Nonactivated and PHA-Activated Normal PMB Target Cells

Donor	PHA*	Fluorescence† HTLV-I/R-16 Cell Bound
1	−	46
	+	21,75
2	−	46
	+	20,90
3	−	46
	+	19,91
4	−	90
	+	19,92

*Both PHA-activated and nonactivated target cells were analyzed in the same experiment on the same day. In the PHA(−) samples, Ficoll-Hypaque density-gradient–purified mononuclear cells were immediately used in the above experiment. In the PHA(+) samples, 5 μg/mL PHA was added to target cell cultures for 24 hours. The cells were washed and resuspended in fresh media with 20% partially purified, delectinated IL 2. At 6 days, the virus adsorption assay was performed.

†Data represent relative mean fluorescence.

Competitive binding of HTLV-II. Table 3 shows the results of cross-interference experiments between HTLV-I and HTLV-II in MT-2 and Mo-T cells initially adsorbed with one HTLV/R-16 and subsequently challenged with a homologous or heterologous HTLV/R-16. In MT-2 cells, HTLV-I/R-16 bound more effectively than HTLV-II/R-16, but the cross-interference patterns were very similar. HTLV-II blocked 66% of HTLV-I/R-16 binding, whereas HTLV-II inhibited 54% of its own adsorption. In Mo-T cells, constitutively producing HTLV-II, the number of HTLV-I binding sites was still higher than for HTLV-II. The homologous interference pattern of HTLV-II in Mo-T cells was slightly less than the homologous interference in MT-2 cells with HTLV-I.

DISCUSSION

In this report, a flow cytometric analysis used in previous Epstein-Barr virus (EBV) cell receptor studies[32] is described for investigation of HTLV-I specific binding to fresh and cultured cells on a single cell basis. This assay used rhoda-

Table 3. Competitive Binding of HTLV-II with HTLV-I

Target Cells	Competitive HTLV* Unlabeled	R-16-Labeled† HTLV	Relative Mean Fluorescence	Fluorescence Reduction (%)
MT-2	—	HTLV-I	66	—
MT-2	HTLV-I	HTLV-I	30	54
MT-2	HTLV-II	HTLV-I	22	66
MO-T	—	HTLV-I	47	—
MO-T	—	HTLV-II	12.5	—
MO-T	HTLV-II	HTLV-II	7.2	43

*Competitive unlabeled HTLV-I or HTLV-II at the same concentration as labeled virus were incubated at 4 °C for 2 hours followed by incubation with HTLV/R-16.

†HTLV-I or HTLV-II purified virions (50 pmoles of reverse transcriptase activity) were labeled with R-16 rhodamine hydrocarbon and incubated at 4 °C for 30 minutes with 7.5 × 10^5 target cells.

mine hydrocarbon-tagged, purified HTLV-I virions, according to the procedure originally published for avian retroviruses.[22,24] Automated detection of HTLV-I binding by flow cytometry permitted rapid, sensitive, and reproducible testing. Accurate quantitation of virus binding to single cells could be performed on as few as 10^4 cells. The rate of virus–cell binding was temperature dependent, characteristic of all virus–cell receptor interactions. Only a specific virus–cell interaction was detected because nonrhodamine-tagged, homologous HTLV-I interfered with HTLV-I/R-16 binding significantly in susceptible but not resistant target cells. In contrast, unrelated, nonlabeled retroviruses were unable to block HTLV-I/R-16 binding in HTLV-I–sensitive target cells. Moreover, several target cell lines were insensitive to HTLV-I/R-16 adsorption.

The existing knowledge regarding the HTLV-I and HTLV-II cell membrane receptors is scarce as opposed to that which is known regarding the unrelated HIV. In contrast, the nature of the HIV-I cell receptor has been identified as an epitope of the CD4+ cell surface phenotype which is characteristically found on normal helper T lymphocytes.[33,34] The HTLV-I and HTLV-II receptors, which are known to differ from HIV-I, are still a mystery, although their distribution has been studied by us and other researchers.[35-37] A summary of our studies in lymphoid and nonlymphoid cells follows. First, a direct relationship was shown between the expression of HTLV-I receptors on lymphoid targets and successful transmission of HTLV-I to the latter by cocultivation with irradiated, HTLV-I producing T lymphocytes. The target lymphoid cells are discussed in descending rank order of specific HTLV-I binding. As expected, HUT 102-B2 and MT-2, HTLV-I constitutively producing T lymphoblast cell lines expressed high levels of HTLV-I binding sites. This, in part, is a consequence of the relative large surface area of these cells. In reality, the number of binding sites may be much higher in these cells because some of the viral binding sites may be masked by previously bound, endogenous budding HTLV-I virions. These observations confirmed earlier studies using a fluoresceinated virus–cell binding assay analyzed on a flow cytometer.[35,36] MOLT 4, is the only immature T cell line identified with HTLV-I binding sites and susceptible to infection. This neoplastic cell line was derived from a patient with acute lymphocytic leukemia. Four other established human T cell leukemic cell lines were studied by Sinangil et al.[36] Two of four carried HTLV-I adsorption sites and were capable of HTLV-I infection. Weiss et al observed HTLV-I cell surface receptor on most T cell lines tested by an indirect VSV (HTLV) pseudotype infectivity assay.[15] Enhancement of HTLV-I binding sites in a subpopulation of the mononuclear targets was noted with mitogen activation and IL 2 stimulation of fresh adult PBMs as compared with nonactivated control PBMs. This pattern was observed in three of four samples tested and in complement studies by Merl et al,[8] who demonstrated that proliferating cycling T cells were more sensitive to HTLV-I transmission and subsequent transformation than were resting, nonstimulated T cells. Sinangil et al observed HTLV-I adsorption sites on fresh adult nonactivated T lymphocytes from a single donor by direct binding assays.[36] All other lymphoid cells examined (including HUT 78, a non–HTLV-I–producing lymphoblast cell line from a cutaneous T cell lymphoma patient; FR45, a continuous normal, EBV-positive B cell line; and NK41, a continuous normal NK T cell line) were relatively resistant to HTLV-I adsorption. These results suggest that the latter target cells either have very low or no HLTV-I binding sites. Our results with the HUT 78 T cells confirm an indirect VSV (HTLV-I) pseudotype transmission analysis.[15] Two other transformed B cell lines were examined previously.[36] The two Burkitt's lymphoma B cell lines, Loukes and Raji, were highly to moderately positive for expression of HTLV-I binding sites without a direct correlation with HTLV-I transmission.[36] Virus transmission may have proved successful if cocultivation with HTLV-I–producing T cells instead of cell-free virus infection had been used or if virus transmission had been examined at a much later time. An alternative explanation would be a postpenetration cellular restriction responsible for blockade of HTLV-I growth.

The relatedness of HTLV-I and HTLV-II envelope glycoproteins and their specific binding sites was also studied by us and by other researchers.[15,18] Sodroski et al compared the primary structure of the exterior spike glycoprotein of HTLV-I and HTLV-II.[18] The major glycoprotein of HTLV-I and HTLV-II showed 65% identity. Within the spike glycoprotein, similarity between the regions of hydrophilicity and hydrophobicity, glycosylation sites, and the location of cysteine residues suggested common secondary and tertiary structures.[18] In addition, limited interference and cross-neutralization of VSV (HTLV-I) and VSV (HTLV-II) pseudotypes were observed,[15] indicating that the envelopes of HTLV-I and HTLV-II are similar but antigenically distinct. Weiss and co-workers studied the relatedness of HTLV-I and HTLV-II envelope glycoprotein cell membrane receptor sites. Both viruses appear to recognize the same receptors, as shown by receptor interference of syncytium induction (cell fusion) and pseudotype infectivity, but HTLV-II induced cell syncytia in a more restricted host cell range.[15] Other differences also existed. Indeed, HTLV-I could be grown in a number of nonlymphoid cell types, whereas attempts to propagate HTLV-II in the same cells were unsuccessful.[15]

The above findings were confirmed here by direct virus-cell binding analyses on the flow cytometer. HTLV-II was highly competitive with HTLV-I for MT-2–specific virus binding sites. HTLV-II bound fewer specific adsorption sites than did HTLV-I. This difference may reflect the difference in adsorption time of the nonlabeled and labeled virus and/or a lesser affinity of HLTV-II for the same receptor sites. Furthermore, there was more HTLV-I and HTLV-II specific adsorption of MT-2 cells than of Mo-T cells, neoplastic T lymphoblasts constitutively releasing HTLV-II. These observations correlate with the fact that MT-2 cells possess more integrated proviral HTLV copies and a faster growth rate than does Mo-T.

In two nonlymphoid, nonhuman cell lines, a direct correlation between HTLV-I adsorption sites and HTLV-I infection could not be established. The cell lines, African green monkey kidney cells (VERO) and wild mouse fibroblasts

(SC-1) bound high levels of HTLV-I virions, but viral transmission was not successful. These data directly indicate the presence of HTLV-I receptors. An indirect pseudotype infectivity assay and syncytia formation assay with T cells producing HTLV-I of 15 different nonlymphoid cells (including VERO) indirectly suggested the same conclusion.[37] These studies suggested that HTLV-I receptors were present on all cells capable of VSV (HTLV-I) pseudotype propagation or cell fusion. Therefore, in nonhuman, nonlymphoid cell cultures, the cellular receptor restriction was not the major determinant of cell tropism of HTLV-I. In conclusion, our findings and further extended studies may lead to a clearer understanding of host susceptibility to disease, the pathogenesis of HTLV-I–induced ATL, and possible modification of host receptors for disease prevention.

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Separation of Human Plasma Factor IX From HTLV-I or HIV by Immunoaffinity Chromatography Using Conformation-Specific Antibodies

By Steven A. Limentani, Barbara C. Furie, Bernard J. Poiesz, Richard Montagna, Keith Wells, and Bruce Furie

Immunoaffinity chromatography using conformation-specific antibodies yields pure factor IX from human plasma in a single rapid, facile purification step. We evaluated this technique to determine whether factor IX can be separated from human T cell leukemia virus-I (HTLV-I) and human immunodeficiency virus (HIV) in plasma supplemented with these viruses. Viral content was determined with an enzyme-linked immunosorbent (ELISA) assay sensitive to 50 ng viral protein. Both HTLV-I and HIV coeluted with unbound protein. Neither HTLV-I nor HIV was detected in purified factor IX. We conclude that, to the limits of detection, factor IX purified by this method is free of viral contamination.

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HEMOPHILIA B is a sex-linked disorder characterized by a functional or quantitative deficiency of factor IX.[1-4] Bleeding episodes are treated with factor IX concentrates containing intermediate purity factor IX.[5-10] These agents are associated with complications, including disseminated intravascular coagulation, thrombotic disorders, hepatitis B, non A-non B hepatitis, and AIDS.[11-26] Currently, factor IX concentrates are heat treated to eliminate human immunodeficiency virus (HIV). Although this method may be effective in reducing HIV, definitive data on the role of heat treatment in preventing AIDS in hemophiliac patients awaits additional epidemiologic data. Furthermore, heat treatment results in the loss of 8% to 40% of the factor IX coagulant activity.[27-34]

We have described a method for the rapid, one-step purification of factor IX from plasma.[35] Antibodies specific for the metal-stabilized conformation of factor IX are used to bind factor IX to an insoluble support. Factor IX is dissociated from the antibodies by the addition of EDTA and the conversion of factor IX to its metal-free conformation.[35] The factor IX isolated by this method is homogeneous and has a specific activity of 120 to 150 U/mg. Furthermore, the factor IX isolated from plasma contaminated with hepatitis B virus yields factor IX free of measurable virus.[35] The purpose of the current study was to determine whether immunoaffinity chromatography using conformation-specific antibodies separates factor IX from HTLV-I or HIV.

MATERIALS AND METHODS

Preparation of HTLV-I and HIV. HTLV-I was produced from the cell line HUT 102 B2,[36] and HIV was produced from the B cell line FCRC-15/AAV.[37] Normal pooled human plasma was obtained from donors who were negative for HTLV-I and HIV antibody and antigen.[36-39] Purified HTLV-I and HIV were prepared by centrifugation (45,000 g) of cell-free conditioned media from the respective cell lines.[36] Pelleted particles were sedimented on alternating discontinuous glycerol (30% over 100% cushion), continuous sucrose (22% to 65%), and discontinuous glycerol (30% over 100% cushion) gradients. Positive fractions were identified by assaying reverse transcriptase activity using the template primer poly (C) oligo dG in the presence of 10 mmol/L $MgCl_2$.

Competitive enzyme-linked immunosorbent assay (ELISA) for HTLV-I and HIV. Polyclonal rabbit anti–HTLV-I and anti-HIV antibodies were raised in rabbits immunized with ether-disrupted purified virions, and the antibodies were measured by ELISA and Western blot assays for HTLV-I and HIV antibodies.[38] Microtiter plates were coated with 200 ng viral protein (either HTLV-I or HIV) in 100 μL CBC (150 mmol/L Na_2CO_3; 350 mmol/L $NaHCO_3$,pH 9.6) and then incubated overnight at 4°C. After incubation, excess solution was removed and 100 μL of 1% bovine serum albumin (BSA) in CBC was added to each well; the plate was incubated overnight at 4°C.

Dilutions of rabbit antisera ($^1/_{60}$ for HTLV-I and $^1/_{5,000}$ for HIV) were made in phosphate-buffered saline (PBS)-Tween 20. Competing proteins and diluted antisera were incubated together for 1 hour at 23°C. The viral protein-coated wells were washed three times with 0.1% BSA in PBS-Tween 20 and then 100 μL of the antiserum mixture described above were added to the appropriate well. After incubation for 1 hour at 23°C, the plates were washed three times with PBS-Tween 20 and 100 μL of goat anti-rabbit IgG alkaline phosphatase conjugate (1/5,000 dilution in PBS-Tween 20) was added. The plates were incubated for 2 hours at 23°C, washed three times with PBS-Tween 20, and 100 μL of *p*-nitrophenyl phosphate was added to each well. The plates were developed for 30 minutes, and the reaction was stopped with 15 μL of 3 N NaOH. The plates were read at 405 nm.

Preparation of anti-factor IX:Mg(II) antibodies. Antibodies were isolated as previously described, except that a subpopulation of conformation-specific antibodies were used that bind to factor IX in the presence of Mg^{2+}. Antibodies that bind to factor IX in the presence of Ca^{2+} but not Mg^{2+} were removed.[40] Rabbit anti-factor IX:Mg(II) antibodies were coupled to cyanogen bromide-activated Sepharose 4B at a concentration of 2.5 mg/mL Sepharose.[41] In the experiment with HIV, factor IX was isolated on the anti-factor IX:Mg(II) antibody–Sepharose column.[40] An anti-factor IX:Ca(II)–Sepharose column was used in the experiment with HTLV-I; this column has been previously described.[35]

Isolation of factor IX from virus-supplemented plasma with anti-factor IX:Mg(II) Sepharose. Pooled human plasma (100 mL) was dialyzed overnight against 0.05 mol/L Tris-HCl, pH 7.4, 0.5 mol/L NaCl. After dialysis, the plasma was adjusted to 7.5 mmol/L

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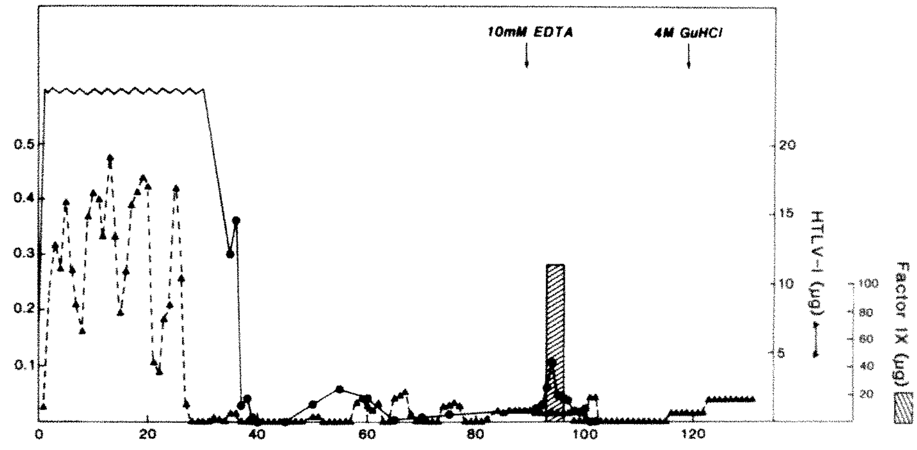

Fig 1. Purification of human factor IX from plasma contaminated with HTLV-I. Elution of protein was evaluated by absorption at 280 nm (●). Elution of HTLV-I was measured using an ELISA directed against the viral coat protein (▲). Elution of human factor IX was assayed by a radioimmunoassay; the total factor IX contained in the pooled fractions is shown (hatched columns).

with 1 mol/L $MgCl_2$. Then either 500 μg of HTLV-I or 500 μg of HIV was added to the plasma. As a control, an equal volume of plasma without added viruses was applied to the affinity column to assess nonspecific background in both the HTLV-I and the HIV assays. The column was developed as previously described except that Tween-20 was added to buffers only in the experiment in which HTLV-I was added to plasma. The flow-through was collected in 5.5-mL fractions initially and then 1.5-mL fractions during elution of factor IX with 10 mmol/L EDTA. The protein concentration was estimated by the absorption at 280 nm and by factor IX radioimmunoassay. Pooled fractions were subjected to high-speed centrifugation (49,000 g) for 60 minutes. The pellets were disrupted with ether and resuspended in 100 μL of PBS-Tween-20. Ten microliters were then assayed for HTLV-I or HIV.

RESULTS

Evaluation of viral content of affinity-purified factor IX. HTLV-I (500 μg) was added to pooled human plasma prior to isolating factor IX on the anti-factor IX:Ca(II)–Sepharose column. HTLV-I coeluted with unbound protein. No HTLV-I was detected in the material that was eluted from the column with either EDTA or guanidine HCl (Fig 1). By this analysis, a minimum of a 1,000-fold reduction in HTLV-I occurred in face of a 10,000-fold increase in the specific activity of factor IX.

In a separate experiment, HIV (500 μg) was added to pooled plasma prior to the application of the plasma to the anti-factor IX:Mg(II)–Sepharose column. The HIV coeluted with unbound protein. No viral contamination was detected in the material eluted from the column with either EDTA or guanidine HCl (Fig 2). As previously, a minimum of a 1,000-fold reduction in HIV occurred in face of a 10,000-fold increase in the specific activity of factor IX.

The standard curves for the competitive ELISA demonstrated a sensitivity to 50 ng viral protein (Fig 3). Pooled human plasma without endogenous or added viral contaminants was applied to the anti-factor IX:Mg(II)–Sepharose column. The unbound and bound fractions obtained from this experiment were assayed for HTLV-I and HIV to determine accurately the level of nonspecific binding in the assay. The OD_{405} values observed were equivalent to the OD_{405} values observed in the factor IX prepared from experimentally contaminated plasma samples.

DISCUSSION

This study examined the behavior of two of the human retroviruses, HTLV-I and HIV, when they are present in plasma used for purification of factor IX. Our data indicate that, to the limits of detection of the immunoassay systems used, these viruses do not copurify with factor IX. The virus

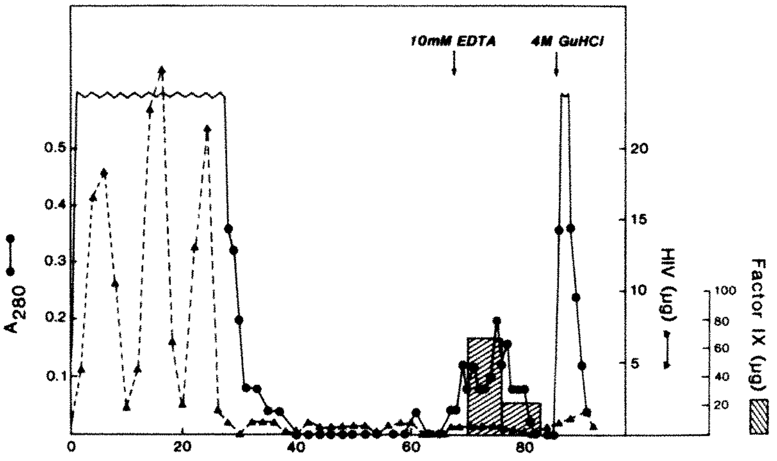

Fig 2. Purification of human factor IX from plasma contaminated with HIV. Elution of protein was evaluated by absorption at 280 nm (●). Elution of HIV was measured using an ELISA directed against the viral coat proteins (▲). Elution of human factor IX was evaluated using a radioimmunoassay; the total factor IX isolated in pooled fractions is shown (hatched columns).

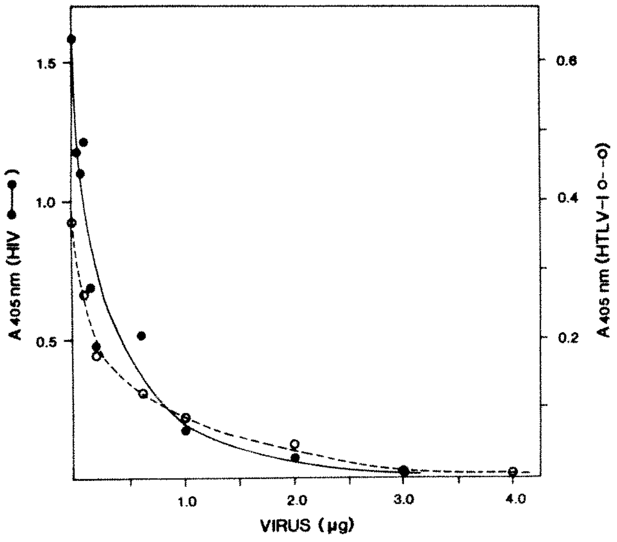

Fig 3. ELISA systems for quantitation of viral proteins from HTLV-I or HIV. HIV (●——●), HTLV-I (○——○).

and the factor IX that is eluted with EDTA are resolved. Our data from these and earlier experiments indicate a general method for the separation of factor IX from viruses using an immunoaffinity purification involving specific binding and specific elution.

The morbidity associated with infusion of currently available factor IX concentrates has been well described. Recent epidemiologic data on the development of the acquired immunodeficiency syndrome in patients with hemophilia are of great concern.[23-26] Thirty percent to 36% of the patients treated with factor IX concentrate showed HIV antibody positivity.[23,24] Lyophilization of factor IX concentrates results in a 10- to 100-fold decrease in virus.[30,34] Heat treatment of factor IX concentrates for 20 hours at 60°C renders HIV undetectable.[27,34] Assumptions regarding the complete sterilization of factor IX concentrates from HIV contamination are done by extrapolation of the data derived from immunoassays.[34] A small group of hemophilia A patients whose sera were initially negative for anti-HIV antibodies have received heat-treated factor VIII concentrate for up to 1 year without seroconverting.[29] This suggests marked improvement can be anticipated in treatment of hemophilia B patients with heat-treated factor IX concentrate. Heat treatment, however, results in the loss of ~8% to 40% of the factor IX coagulant activity of the factor IX concentrate.[32,34]

Our technique provides a rapid, one-step quantitative purification of homogeneous factor IX. We showed that, to the limits of detection, the factor IX isolated by this method is free of retrovirus, even when these viruses are added in high titer to plasma. These results indicate that this immunoaffinity method might be integrated into methods of manufacture of factor IX used for the treatment of hemophilia B. Whether this method can eliminate the need for heat treatment of plasma to eliminate HIV or render the factor IX free of hepatitis viruses will require direct infectivity assays or systematic study of patients treated with this new preparation.

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Flow Cytometric Analysis of Human Bone Marrow. II. Normal B Lymphocyte Development

By Michael R. Loken, Virendra O. Shah, Karen L. Dattilio, and Curt I. Civin

A panel of B lymphoid-reactive monoclonal antibodies was used to analyze the phenotypic changes that accompany B lymphocyte development in normal human bone marrow. The B lymphoid cells were identified using light scattering and the expression of CD19 on a flow cytometer. Quantitative three-color immunofluorescence was then used to correlate other cell surface antigens on these cells identified as B lymphoid in normal marrow. CD10 and CD20 identified almost exclusive populations and provided a convenient means of discriminating between the less and more mature B lineage cells. The CD10^{+} cells could be further subdivided using CD34. The population of CD19^{+}, CD10^{+}, CD34^{+} cells comprised only 0.6% of marrow cells, but these contained the majority of terminal deoxynucleotidyl transferase (TdT^{+}) cells. In the assessment of class II antigens, HLA-DR was expressed on all B lineage cells whereas HLA-DP preceded HLA-DQ in appearance during the developmental process. Among the later antigens expressed on B lineage cells, cell surface IgM, CD20, and HLA-DQ were expressed at essentially the same time. Cell surface CD10 was lost at the time when CD21 and CD22 were acquired on the cell surface. These data illustrate that multiparameter flow cytometry can be used to define a continuous progression of stages of B lymphocyte development based on cell surface antigen expression even though these cells represent a minor fraction of normal marrow cells.

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THE BONE MARROW is the site of production and early maturation of B lymphocytes in humans.[1] A correlation between maturational stage and cell surface antigen expression of B lymphocytes has been made, primarily on the basis of cell surface phenotypes of acute lymphoblastic leukemias (ALL) and lymphomas.[2-4] Counterparts of many of the malignant cells have been identified in normal bone marrow.[1,5-7]

Previous studies of B lymphocyte surface antigens have identified CD19 (Leu-12, B4) as a marker for all B lymphocytes.[8,9] CD19 is expressed very early in B cell development and is retained until the latest stages of cell activation and antibody secretion.[8] This marker is expressed on most non-T ALL (>95%), but is not found on T cell ALL or on myeloid lineage leukemias.[2,3] Thus, CD19 defines most cells of the B lineage with exceptional lineage specificity.[2]

Another B cell antigen, CD20 (Leu-16, B1), is expressed later during B lymphocyte development.[10,11] As compared with CD19, CD20 is expressed on fewer normal B lymphocytes in the marrow and on a lower percentage of leukemias.[2,3,10,11] CD10 (common ALL antigen, CALLA, J5) has been found on most cases of non-T ALL but is expressed on only a subset of immature B lymphocytes.[2,12,13] Mature peripheral blood B lymphocytes express both CD19 and CD20, but do not express CD10.[10,12] The progenitor cell-associated antigen, CD34 (My10) is also observed on some B lineage ALL cases, but is not expressed on normal peripheral blood B cells.[14]

Mature B lymphocytes in peripheral blood express class II antigens including HLA-DR, HLA-DP, and HLA-DQ, as well as several other B lymphocyte specific antigens: CD21 (CR$_2$), CD22 (Leu-14), cell surface IgM (sIgM), and IgD (sIgD).[15-19]

In this article, we correlate the expression of multiple cell surface markers on normal bone marrow B lineage cells. By using multiparameter quantitative analysis on a flow cytometer, we examined all low-buoyant-density cells obtained from the bone marrow sample without preenrichment. The entire B lymphoid lineage was identifiable using CD19 in combination with light-scattering characteristics. Pairs of other cell surface antigens were then identified and correlated on the CD19^{+} cells by using three-color immunofluorescence.[20] From these data in combination with cell sorting and immunostaining for terminal deoxynucleotidyl transferase (TdT), we could define the sequence of appearance during B lymphocyte development of these antigens: HLA-DR, HLA-DP, HLA-DQ, CD10, CD19, CD20, CD21, CD22, CD34, sIgM, sIgD, and nuclear TdT.

MATERIALS AND METHODS

Monoclonal antibodies. Fluorescein-conjugated (FITC) and/or phycoerythrin (PE)-conjugated CD3(Leu-4), CD10(CALLA), CD11b(Leu-15), CD14(Leu-M3), CD16(Leu-11), CD19(Leu-12), CD20(Leu-16), CD21(CR$_2$), CD22(Leu-14), anti-HLA-DR, anti-HLA-DP, anti-HLA-DQ, streptavidin allophycocyanin (APC) along with purified CD34(HPCA-1) were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. Anti-glycophorin (10F7) was a kind gift from Dr R. Langlois (Lawrence Livermore National Laboratory, Livermore, CA) CD19(Leu-12) and CD10(CALLA) (Becton Dickinson) were coupled with biotin, following the protocol of Billingsley.[21] Anti-human IgM-FITC, anti-human IgD-FITC and F(ab')$_2$-goat anti-mouse Ig-FITC were obtained from Tago, Burlingame, CA.

Cell preparation and immunofluorescence. Bone marrow aspirates were obtained from consenting normal adult volunteers. Collection of samples was performed after being approved by the Institutional Review Board, as approved by the Department of Health and Human Services. The low-density leukocytes (1.077 g/cm^3, Ficoll-Hypaque, Pharmacia, Piscataway, NJ) were washed

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in RPMI 1640 containing 10% fetal bovine serum (FBS) and aliquoted for immunofluorescent staining. The cells were suspended at a concentration of 10^7/mL. In the experiments in which both antibodies were directly conjugated to the fluorochrome, the first antibody was added to 10^6 cells using an amount of antibody that gave maximal fluorescence. The cells were incubated on ice for 20 minutes. The cells were then pelleted and washed with buffer (at least five times the staining volume) before the second antibody was added. After 20 minutes on ice, the cells were again washed with buffer.

For three-color analysis, cells were reacted with FITC-conjugated, PE-conjugated, and biotin-conjugated antibodies for 20 minutes and were then washed. APC-conjugated streptavidin was then added, and the cells were incubated for an additional 20 minutes and washed and fixed in 1% paraformaldehyde.

Three-color analysis using CD34 was performed as follows: Cells were incubated with CD34 on ice for 20 minutes, washed and incubated with $F(ab')_2$ goat anti-mouse Ig for 20 minutes, and washed. Cells were further incubated with 10% normal mouse serum to block any free binding sites of the second step antibody. After being washed, the cells were reacted with PE-conjugated and biotin-conjugated antibodies, followed by the APC-conjugated streptavidin. Unstained cells, IgG1-FITC, IgG2-PE, and cells labeled with streptavidin-APC but without biotin conjugate were included as controls.

Flow cytometry. Quantitative fluorescence analysis was performed using a five-parameter FACS 440 (Becton Dickinson Immunocytometry Systems). The optical configuration and filter combinations for three-color immunofluorescence using FITC, PE, and APC have been described previously.[20,22] The argon-ion laser was operated at 488 nm using 200 mW of power. FITC emissions were collected with a 530/30 bandpass (BP) filter, and PE emissions were collected with a 585/42 BP filter. The laser beam was focused using a 2-inch focal length spherical lens. Three-color immunofluorescence was performed using HeNe laser of 633 nm, 40 mW, together with an argon laser as described.[23] APC emission was collected with a 660/20 BP filter. Fluorescence signals were processed using a four-decade logarithmic amplifier. Light scattering signals were processed by linear amplification.

A minimum of 30,000 events for each sample was collected in list mode on a Consort 30 Data Management System, Becton Dickinson. This permitted reanalysis of the data to demontrate the correlation between three colors of immunofluorescence for populations of cells identified by forward-angle and right-angle light scattering. In the assessment of class II and later B lymphoid antigens of the marrow B lineage cells, the data were pregated on light scattering and CD19 fluorescence. The contour lines in Figs 1 through 7 were drawn at levels of 2, 4, 8, 16, 32, 64, and 128. The fluorescence correlations in Figs 2 through 7 were gated on the light-scattering window depicted in Fig 1A.

Cell sorting. Viable cells, stained for two colors of immunofluorescence, were sorted based on the gates indicated in the appropriate Figs. Replicate cytocentrifuge slides were prepared for each sorted fraction of cells. These slides were coded, stained for nuclear TdT (rabbit anti-TdT, a kind gift from Dr F. Bollum) using an immunoperoxidase procedure (Supertechs, Bethesda, MD) or Wright's, and differential counts were made for each sample.[24]

RESULTS

Bone marrow B lymphocytes have distinctive light-scattering properties. Normal human bone marrow cells were heterogeneous in their forward- and right-angle light-scattering characteristics (Fig 1A). The $CD19^+$ B cells were confined to a well-defined cluster, which was previously classified as "lymphoid" (Fig 1B) with low-to-moderate forward-angle and low right-angle light-scattering properties.[25] All subsequent multicolor immunofluorescence analyses were performed by gating on this light-scattering population, as shown in the box in Fig 1A. More than 95% of the $CD19^+$ cells in whole ungated marrow were included within this gate.

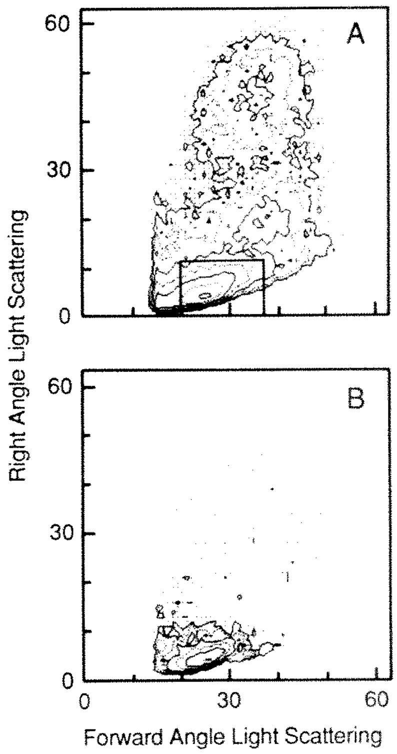

Fig 1. The correlation between forward- and right-angle light-scattering for (A) all low-buoyant-density bone marrow cells and (B) the CD19/Leu-12$^+$ B lineage cells. The box in A identifies the gate used to reanalyze the list mode data. This gate included >95% of all CD19$^+$ cells. A minimum of 30,000 events were collected for each list mode file.

We used two-color immunofluorescence to confirm that CD19 bound only to B lineage cells and not to cells of other lineages in normal human bone marrow. Normal marrow cells were reacted with CD19 in combination with either CD3 (T cells), CD16 (natural killer [NK] and neutrophils), CD14 (monocytes), CD11b (NK, monocytes and neutrophils), or anti-glycophorin (erythroid cells).[26-30] Quantitative fluorescence analysis on the flow cytometer demonstrated that the cells labeled with CD19 were not double labeled with the other antibodies (data not shown); thus, CD19 and the other antibodies identified mutually exclusive cell populations in bone marrow.

CD20 and CD10 define distinct stages of B lymphocyte development. The CD20 antigen is expressed late during B lymphocyte development, whereas CD10 has been associated with early B lymphocytes.[10-13] To define more precisely when, during development, B lymphocytes lose CD10 and acquire CD20, we performed three-color immunofluorescence experiments using CD10 and CD20 in correlation with CD19.

The two-color correlated expression of CD20 and CD19

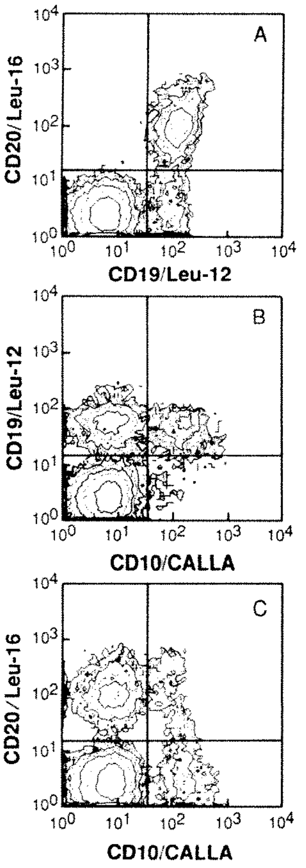

Fig 2. Three-color immunofluorescence analysis of bone marrow cells labeled with CD19/Leu-12 PE, CD20/Leu-16 FITC, and CD10/CALLA biotin (APC-streptavidin). Correlations were made between (A) CD19 and CD20, (B) CD19 and CD10, and (C) CD10 and CD20. All data were gated on the light-scattering window in Fig 1A. Although both the CD20$^+$ and the CD10$^+$ cells were also CD19$^+$ (A, B), they identified almost completely non-overlapping populations (C). The quadrants were set using appropriate negative controls.

on marrow cells (Fig 2A) confirmed that CD20 was expressed on a subset of the CD19$^+$ cells; all CD20$^+$ cells were also CD19$^+$. No CD20$^+$ cells were outside the lymphoid light scattering window.

CD10 was also found on a portion of the CD19$^+$ cells (Fig 2B). CD10$^+$, CD19$^-$ marrow cells were identified but these cells fell outside the lymphoid light-scattering window and were granulocytes (data not shown).

The correlation between CD20 and CD10 (Fig 2C) indicated that these two antibodies identified almost non-overlapping B lymphoid subpopulations: Most CD20$^+$ cells did not express CD10 and vice versa. A small subset of cells, however, expressed both markers. The proportions of cells within marrow that expressed CD19, CD20, and CD10 are presented in Table 1. CD19 stained ~6.5% of all marrow cells, whereas CD20 labeled ~4.5%. A slightly smaller proportion of marrow cells, 3.5%, were CD10$^+$. Of the CD19$^+$ B cells in marrow, ~60% expressed CD20, whereas only 40% expressed CD10. Only ~20% of the CD20$^+$ cells were also CD10$^+$, and a similar proportion of all the CD10$^+$ cells in this light-scattering window were double labeled with CD20. Together, CD10 and CD20 identify almost all CD19$^+$ B lineage cells. A few marrow B lineage cells (<5% of CD19$^+$ cells) lack both CD10 and CD20, a phenotype consistent with plasma cells.

Early marrow B lineage cells co-express CD19, CD10, and CD34. CD34 was expressed on a subset of B lineage ALL cases as well as on a small population of immature normal marrow cells, including all in vitro assayed hematopoietic progenitor cells, but was not expressed on mature normal cells.[14] All TdT$^+$ cells in normal marrow were previously found in the CD34$^+$ fraction.[31] Because TdT expression is a characteristic of early lymphoid cells, we used three-color immunofluorescence to investigate whether the B cell antigens identified by CD19 and CD10 could be found on any CD34$^+$ cells.

In the correlation between CD19 and CD34, a small population of dim CD19$^+$ cells was also labeled with CD34 (Fig 3A). Most of the CD19$^+$ cells, however, were negative for the CD34 marker (Table 2). Less than one-half of the CD34$^+$ cells were CD19$^+$.

In a similar correlation between CD10 and CD34, a population of cells was double labeled. The cells that were most intensely stained with CD10 were the cells that also bound CD34 (Fig 3B). In three-color experiments, these few CD10$^+$, CD34$^+$ cells were the ones that also expressed low amounts of CD19 (Fig 3A). These CD34$^+$, dim CD19$^+$, bright CD10$^+$ cells constituted <1% of total marrow cells (Table 2).

To characterize this minor cell population further, marrow cells were reacted with either (a) CD34 and CD19, or (b) CD34 and CD10. The cell subpopulations identified were then (FACS) sorted, and cytocentrifuge slides were prepared from the sorted fractions (Fig 3A and B shows sorting windows). These slides were examined for morphology (Wright's stain) or for nuclear TdT expression[24,31] (Table 3).

Table 1. Proportion of B Lymphoid Populations Identified by CD19, CD20, CD10 in Normal Bone Marrow

		Mean Percentage of Positive (SD,n)*		
Cell Surface Phenotype†	Total Marrow Cells (%)‡	CD19$^+$ Marrow Cells (%)§	CD20$^+$ Marrow Cells (%)‖	CD10$^+$ Marrow Cells (%)¶
CD19$^+$	6.5 (4.3; 53)	NA**	NA	NA
CD20$^+$, CD19$^+$	4.7 (2.9; 29)	59.1 (20.0; 8)	NA	NA
CD10$^+$, CD19$^+$	3.1 (3.1; 15)	38.6 (17.9; 15)	NA	NA
CD10$^+$, CD20$^+$	0.9 (1.0; 9)	10.3 (8.1; 9)	22.0 (12.3; 9)	20.0 (8.1; 9)

*Percentages are reported with (in parentheses) SD and total number of independent samples (n) used in the calculation.

†Cell surface phenotypes were determined using the monoclonal antibodies discussed in the Materials and Methods section.

‡Mean percentage of positive cells calculated for all nucleated cells (30,000) in each marrow sample.

§Mean percentage of positive cells calculated as a percentage of CD19$^+$ cells in each sample.

‖Mean percentage of positive cells calculated as a percentage of CD20$^+$ cells in each sample.

¶Mean percentage of positive cells calculated as a percentage of CD10$^+$ cells in each sample.

**Not applicable.

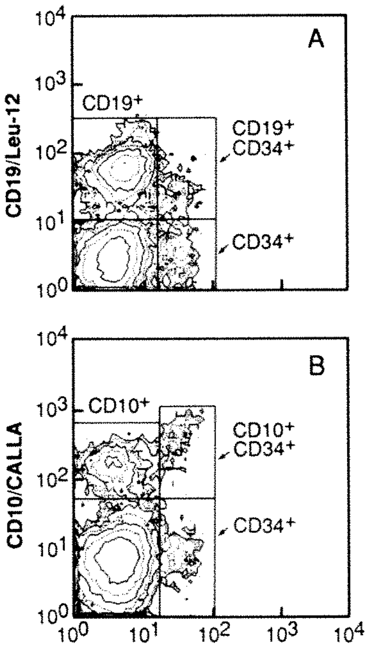

Fig 3. Immunofluorescence analysis of bone marrow cells labeled with CD34/HPCA-1 and either CD19/Leu-12 or CD10/CALLA. (A) The correlation between CD19 and CD34 indicates that a few double-labeled cells were identified. (B) A similar correlation between CD10 and CD34 also showed a double-labeled population of cells. Boxes in A and B illustrate the windows used for sorting the cell populations assessed for morphology and TdT expression (Table 3).

The most immature cells by morphology were in the CD34$^+$, CD19$^-$, CD10$^-$ fraction. This population contained many "undifferentiated" blast cells, without definite lymphoid features. A minor percentage of these cells stained perceptibly for intranuclear TdT. The double-labeled CD34$^+$, CD10$^+$ or the CD34$^+$, CD19$^+$ cells were somewhat more mature by morphology and could be characterized as lymphoblasts or lymphocytes. Most of these cells were intensely labeled (intranuclear pattern) with the TdT stain (Table 3). In contrast, the CD34$^-$, CD10$^+$, or CD34$^-$, CD19$^+$ cells contained a greater number of mature lymphocytes recognized by morphology that were negative for TdT staining.

The cells that did not stain with either set of antibodies (ie, double negative, CD34$^-$, CD10$^-$; or CD34$^-$, CD19$^-$) were a mixture of lymphocytes and developing erythroid cells from the middle to late normoblast stage.[25] These cells did not contain detectable intranuclear TdT.

Table 2. Early B Lymphoid Subpopulations in Normal Human Marrow

Cell Surface Phenotype	Mean Percentage of Positive (SD; n)*		
	Total Marrow Cells (%)	CD19$^+$ Marrow Cells (%)	CD10$^+$ Marrow Cells (%)
CD34$^+$	1.6 (1.0; 27)	NA	NA
CD34$^+$, CD19$^+$	0.8 (0.8; 18)	7.8 (3.9; 13)	NA
CD34$^+$, CD10$^+$	0.6 (0.4; 6)	NA	13.8 (1.1; 6)

*Percentages, SD, and number (n) of samples in calculation as described in Table 1.

Abbreviation: NA, not applicable.

Table 3. Phenotype, Morphology, and TdT Expression of Early B Lineage Cells in Normal Bone Marrow

Cell Surface Phenotype*	TdT Positive (%)†		Morphology‡
	Experiment 1§	Experiment 2	
CD34$^+$, CD10$^-$	1	2	Blast, lymphoblast
CD34$^+$, CD10$^+$	64	81	Lymphoblast, lymphocyte
CD34$^-$, CD10$^+$	1	2	Lymphocyte, lymphoblast
CD34$^-$, CD10$^-$	0	0	Lymphocyte normoblast
CD34$^+$, CD19$^-$	4	4	Blast, lymphoblast
CD34$^+$, CD19$^+$	30	60	Lymphoblast, lymphocyte
CD34$^-$, CD19$^+$	0	2	Lymphocyte, lymphoblast
CD34$^-$, CD10$^-$	0	1	Lymphocyte, normoblast

*Cells of each phenotype (Fig 3) were sorted and cytocentrifuge slides were prepared.

†Cytocentrifuge slides were stained for nuclear TdT as described in the Materials and Methods section.

‡Replicate slides were Wright's-stained and examined for morphology. The predominant morphological type is given first for each category. When blast cells with definite lymphoid features were observed, these were enumerated. Furthermore, morphological distinction among blast cells was not attempted.

§Two complete experiments were performed.

To determine whether CD10 was expressed before CD19 on these early B lineage cells, the correlation of these two antigens on only the CD34$^+$ cells was determined (Fig 4). Although a few cells (~0.1%) expressed CD34 and CD10 without CD19, an equivalent number were identified expressing CD34 and CD19 without CD10 (Fig 4B). Three replicate experiments gave the same results with no clear evidence for either CD10 or CD19 to precede the other on the CD34$^+$ cells.

HLA-DR, HLA-DP, HLA-DQ class II antigens are sequentially expressed during B lymphocyte development. Two-color immunofluorescence studies have shown that normal marrow CD34$^+$ cells express low but detectable amounts of HLA-DR.[32] Quantitative studies of HLA-DR on B cells in comparison to the CD34$^+$ cells indicated that the amount of HLA-DR expressed on the (average) CD19$^+$ cell was approximately four times greater than that on the (average) CD34$^+$ cell (Fig 5). All the CD10$^+$ lymphoid cells expressed HLA-DR, although the brightest CD10$^+$ cells (ie, the CD34$^+$, bright CD10$^+$ from above) were less intensely labeled with HLA-DR than were most CD10$^+$ cells.

Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells.[15,16] Marrow cells were labeled with CD19, CD10, and either anti–HLA-DP or anti–HLA-DQ. By gating on the CD19$^+$ cells, the correlation between CD10 and the class II antigens was assessed solely on the CD19$^+$ B lymphocytes without contamination

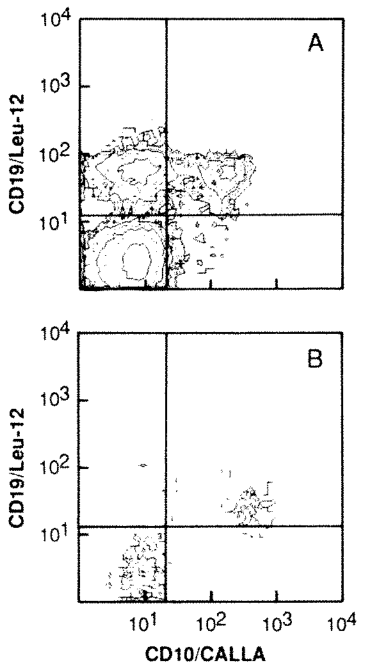

Fig 4. Three-color fluorescence analysis of bone marrow cells labeled with CD19, CD10, and CD34. (A) Correlation between CD19 and CD10 gated on the $CD34^-$ lymphocytes. (B) Correlation between CD19 and CD10 gated on the $CD34^+$ lymphocytes. (The composite ungated display shown in Fig 2B can be compared.)

of other cell types. (Additional class II-bearing cells were identified outside the light-scattering window in Fig 1A, since HLA-DR, HLA-DP, and HLA-DQ also labeled blasts and monocytes within the marrow.) A comparison between Fig 6A and C indicates that HLA-DP was expressed on almost all B lymphocytes, including the $CD10^+$ cells, whereas HLA-DQ stained mainly the $CD10^-$ B lymphocytes. The brightest $CD10^+$ cells (again corresponding to the $CD34^+$, $CD10^+$ cells shown above) lacked both HLA-DP and HLA-DQ.

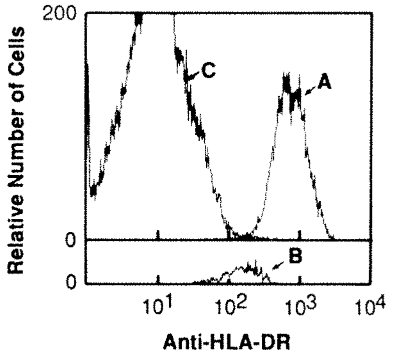

Fig 5. Intensity of HLA-DR expression on lymphoid populations in bone marrow. (A) Histogram of HLA-DR fluorescence gated on $CD19^+$ lymphocytes. This gate includes the $CD10^+$ and $CD20^+$ but not the $CD34^+$ cells. (B) HLA-DR fluorescence histogram gated on the $CD10^+$ bright, $CD34^+$ cells. The axes for this curve have been shifted down to show this minor population of cells on the same relative vertical scale. These cells constitute ~10% of all $CD19^+$ cells in the marrow and express approximately four times less HLA-DR. The remainder of the $CD34^+$ cells also express this lower HLA-DR intensity.[32] (C) Histogram of HLA-DR reactivity on the $CD19^-$ lymphocytes identified by light scatter in the marrow.

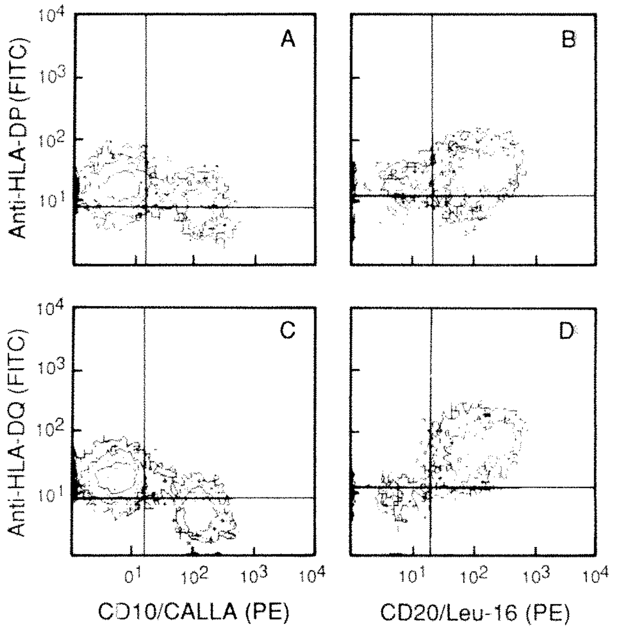

Fig 6. Analysis of class II antigens on marrow B lineage cells. B lineage cells were identified using light scattering and CD19/Leu-12 biotin (APC streptavidin). Only the $CD19^+$ cells (30,000 gated events) were then examined for the correlation between (A) anti–HLA-DP FITC and CD10/CALLA PE, (B) anti–HLA-DP FITC and CD20/Leu-16 PE, (C) anti–HLA-DQ FITC and CD10/CALLA PE, and (D) anti–HLA-DQ FITC and CD20/Leu-16 PE. Quadrants were set using appropriate negative controls.

In reciprocal experiments, marrow cells were labeled with CD19, CD20, and either anti–HLA-DP or anti–HLA-DQ to assess class II expression on more mature marrow B cells. In relating the CD20 with class II antigens on only the $CD19^+$ B lineage cells (Fig 6B and D), HLA-DP reacted with some B lineage cells that were $CD20^-$. (These cells were $CD10^+$, as shown in Fig 6A.) When CD20 and HLA-DQ were compared on the $CD19^+$ cells (Fig 6D), all $CD20^+$ cells were also $HLA\text{-}DQ^+$ and vice versa.

Later stages of B lymphocyte development are defined by CD20, CD22, CD21, sIgM, and sIgD. We used three-color immunofluorescence to study the expression of other cell surface markers on these $CD19^+$ marrow B lineage cells in a pairwise fashion. The data from 1 of 12 similar experiments yielding identical results are shown in Fig 7. Comparing CD20 and sIgM on the $CD19^+$ cells, we found that these two markers identified the same populations (Fig 7A). However, in a correlation between CD20 and sIgD, a significant population of $CD20^+$, $sIgD^-$ cells was identified in addition to the $CD20^+$, $sIgD^+$ cells (Fig 7B).

In the correlation of CD20 with CD22 (Fig 7C), there was a population of $CD20^+$, $CD22^-$ cells, in addition to the $CD20^+$, $CD22^+$ cell population. Likewise, there was a significant $CD20^+$, $CD21^-$ population in addition to a population of $CD20^+$, $CD21^+$ cells (Fig 7I). There were clearly more B lineage cells in the marrow labeled with CD20 than were reactive with antibodies detecting sIgD, CD21, or CD22.

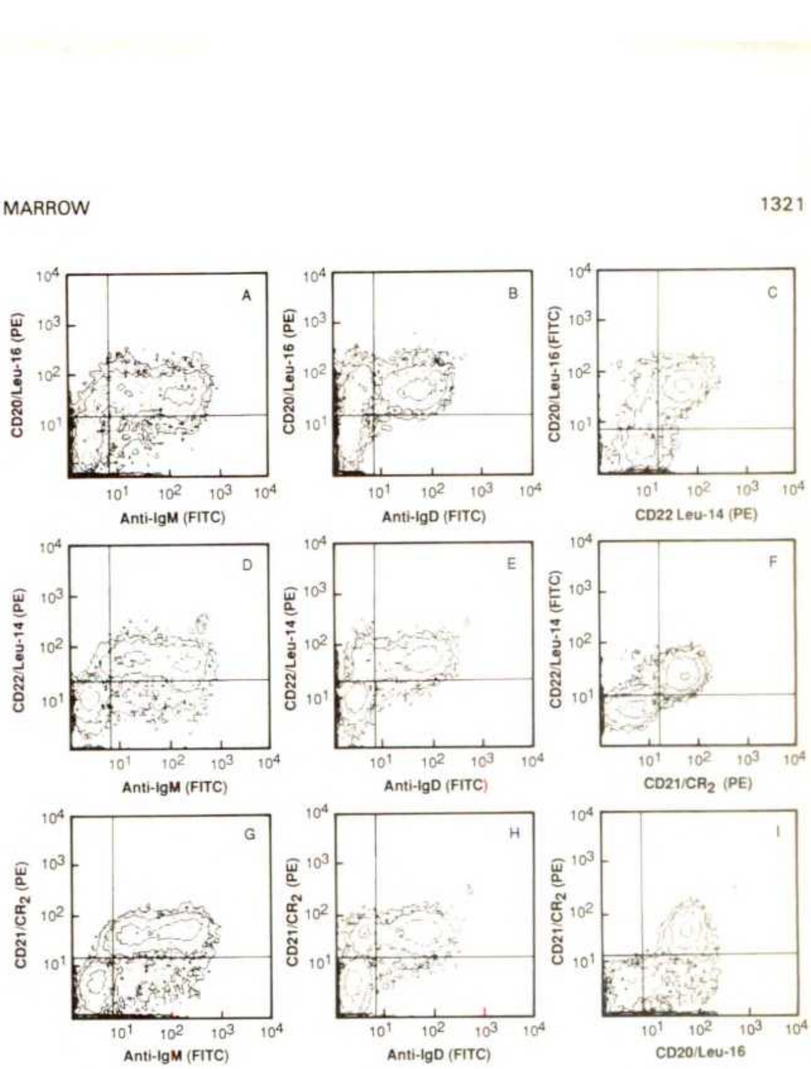

Fig 7. Correlated expression of anti-IgM, anti-IgD, CD20/Leu-16, CD21/CR$_2$, and CD22/Leu-14 on marrow B lineage cells. Only the CD19$^+$ cells (30,000 gated events) were analyzed for the correlation of these five B lymphoid cell surface antigens.

In correlating sIgM with CD22 and CD21 (Fig 7D and G), sIgM was clearly expressed on more marrow B lineage cells than was either CD21 or CD22. When CD22 and CD21 binding was correlated on marrow B lineage cells, the cells identified the same population (Fig 7F). Among the marrow B lineage cells, fewer cells were labeled with sIgD than with either CD20, CD21, or CD22 (Fig 7B, E and H).

DISCUSSION

Adult bone marrow is a complex tissue that is heterogeneous with respect to the multiple lineages represented in a marrow aspirate as well as to maturational differences within each lineage. By identifying cells of the B lineage as distinct from cells of all other lineages, we were able to assess the expression of multiple cell surface markers on cells that differed only in their degree of maturity. The appearance of these cell surface antigens during the maturational process was deduced by comparing in a pairwise fashion the populations labeled by the antibodies.

In these studies, the B lymphoid cells were identified by a combination of light-scattering characteristics (forward and right angle) and the binding of CD19. The light-scattering window used in these studies included essentially all CD19$^+$ cells but excluded cells of both the monocytic and granulocytic lineages.[25] The light-scattering gates were necessary, since the autofluorescence of myeloid cells interfered with the discrimination of the minor subpopulations identified by immunofluorescence.[32] Two-color immunofluorescence showed that CD19 did not label either mature or immature cells of the myeloid lineages. The CD19$^-$ cells classified in this light-scattering window could all be identified as either T, NK, or erythroid cells.[25] In addition, all cells that bound CD19 were identifiable as lymphocytes by morphology. These data are consistent with the studies of non-T ALL in which CD19 was identified as the most precise available antigenic marker of the B lineage.[2]

Three-color immunofluorescence was used to identify the cell surface expression of antigens on the B lymphoid cells in marrow; one color identified the CD19$^+$ B lymphoid cells, and the other two colors compared pairwise the reactivity of additional antigens. The CD19$^+$ population was split into two, almost non-overlapping fractions by the combination of CD10 and CD20. The more mature B lineage cells were identified by the CD20 antigen, whereas the immature B lymphoid cells were CD10$^+$. The CD19$^+$ cells were essentially all double labeled by either CD10 or CD20, suggesting that all the B lymphoid cells were identified in these two populations.

The immature CD10$^+$ B lymphoid cells could be further subdivided by using CD34. An average of 0.6% of marrow cells coexpressed CD19, CD10, and CD34. As found by Ryan and co-workers using lymphocyte-enriched fractions from normal marrow, CD10$^+$ expression was brighter on the CD34$^+$ cells as compared with the remaining CD10$^+$ lymphocytes.[5] Most cells within this population were positive for nuclear TdT.

Class II antigens were not uniformly expressed on marrow B lymphoid cells. HLA-DR was found on all CD19$^+$ cells in marrow but was significantly dimmer on the bright CD10$^+$, CD34$^+$ cells as compared with the more mature CD20$^+$ cells. HLA-DP was not expressed on the brightest CD10$^+$ cells but was found on all other B lymphoid cells. In contrast, HLA-

DQ was found only on the CD20$^+$ cells and not on the less mature CD10$^+$ cells.

The B lymphoid antigens expressed at later stages of maturation reacted only with the CD20$^+$ cells in marrow. Cell surface IgM and HLA-DQ identified the same populations as CD20. In contrast, CD20 reacted with more B lymphoid cells than did CD21, CD22, and anti-sIgD, suggesting that CD20 is expressed before these other antigens.[18]

The data identifying the antigens expressed on B lineage cells can be used to develop a model for the sequential acquisition of cell surface antigens during B lymphocyte development (Fig 8). Our data indicate that the sequence of display of cell surface antigens is highly controlled during development of B cells, with coordinated expression of multiple cell surface antigens at distinct stages during the maturational process. These stages (I, II, III, and IV) are each identified by three or more essentially simultaneous changes in cell surface phenotype.

The most immature cells identifiable in bone marrow express CD34 as well as HLA-DR.[32] CD34 antigen is found on all assayed progenitor cells including CFU-"blast," pre-CFU-GEMM, CFU-GEMM, CFU-GM, CFU-MIX, BFU-E, and CFU-E.[14,33] In the current studies and those of Ryan et al, CD34 is found on the earliest recognizable B lineage cells (CD19$^+$, bright CD10$^+$).[5] This evidence links the CD34 progenitor cell antigen to the B lymphocyte lineage, as well as to the myeloid lineages. HLA-DR was found on these earliest B cells, another characteristic shared by erythroid and myeloid progenitor cells.[32] At this early CD19$^+$, CD34$^+$, HLA-DR$^+$ B cell stage, nuclear TdT and cell surface CD10 are intensely expressed. Because of the presence of TdT in this CD19$^+$, CD34$^+$, bright CD10$^+$ fraction of cells, we speculate that the rearrangement of Ig genes occurs at this stage.[34]

At the beginning of stage II, maturing B lineage cells lose cell surface CD34 and nuclear TdT. At this time, the density of HLA-DR increases fourfold, while the amount of CD10 decreases by a factor of 3. These changes occur with no detectable change in cell size (as assessed by forward light scattering). HLA-DP is detected on cells at approximately this same time.

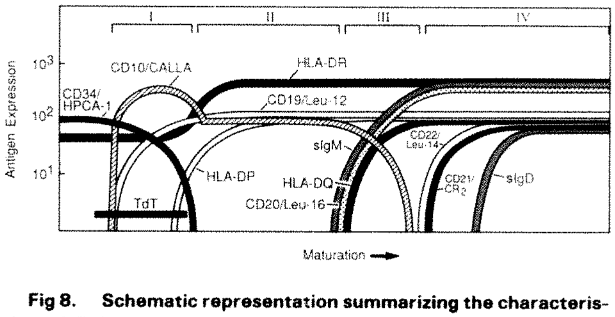

Fig 8. Schematic representation summarizing the characteristics of B lineage cells as they mature in normal human bone marrow. Relative quantities of the cell surface antigens are shown on the abscissa; relative maturation of the cells increases is shown along the ordinate. Four stages of development (I through IV) can be assigned based on multiple changes in cell surface antigen expression that occur at essentially the same time during the maturational process.

The beginning of stage III is marked by the acquisition of CD20, HLA-DQ, and sIgM. The intensity of staining of cells for sIgM, CD20, and HLA-DR is brighter than for HLA-DP, HLA-DQ, or CD19.

The acquisition of two more cell surface markers, CD21 and CD22, and the loss of CD10 identify stage IV. These newly acquired cell surface antigens are not brightly labeled, being expressed in similar amounts as CD19 or HLA-DP. Studies of B lymphoid antigens on peripheral blood indicate that sIgD is expressed on significantly fewer cells than any of the other antigens examined (M.R. Loken and V.O. Shah, unpublished observations). This may explain the data observed in Fig 7B, E, and H and is consistent with stage IV representing peripheral blood contamination of the bone marrow sample. The sIgD$^-$ cells are depicted as more immature cells (as compared with the sIgD$^+$ cells) in Fig 8 to correspond to studies on fetal tissue.[35] Because sIgD is lost after antigenic stimulation, however, the sIgD$^-$ cells observed in blood and marrow may represent the cells that have already been exposed to antigenic stimulation.[36]

The scheme presented in Fig 8 assumes a linear sequence of events in the development of B lymphocytes. The mature B lymphocyte population, as identified in peripheral blood, is assumed to be homogeneous. The lack of expression of sIgD on all peripheral blood B lymphocytes indicates that this assumption is not entirely valid. Other studies must be completed to define where different sublineages of B lymphocytes may deviate from the linear model.

The developmental scheme in Fig 8 is inferred from coordinated expression of cell surface antigens on single samples. Confirmation of the progression from one stage to the next requires the isolation of a particular stage with subsequent induction to the next stage in vitro.

There are differences between the stages of B lymphocyte development identified in this study and those deduced from studies of non-T cell ALL by Nadler and co-workers.[2,3] The most notable difference is in the conclusion from the leukemia studies that CD19 precedes the acquisition of CD10. Nadler et al identified a small group of ALL cases expressing CD19$^+$ and HLA-DR$^+$ but lacking CD10$^-$. In contrast, in our studies on normal B cells, detailed analysis of the CD34$^+$ cells for CD10 and CD19 expression indicated that these antigens were expressed at essentially the same time.[2,3] Similar conclusions were drawn from the studies of Ryan et al.[5] Whether the leukemic cells classified in this group lost the CD10 antigen during the development of the leukemia or if the normal counterpart of this cell is so infrequent (<2% of marrow B cells) that it was not detectable in our studies is not known.

A second difference between our model and that of Nadler et al is that the frequencies of non-T cell ("B lineage") ALL cases classified in the four stages do not appear to reflect the relative proportions of similar populations observed in normal marrow.[3] Almost one-half of the B lineage leukemias had the CD19$^+$, CD10$^+$, CD20$^+$ phenotype, whereas this phenotype constitutes a minor group of normal cells. Although this may be the result of relatively small numbers of patients studied, it is also possible that the leukemic event does not occur randomly during the developmental process and that the proportion of leukemias classified into each group does not reflect the proportion of the normal counterpart in the bone marrow. A third explanation may come from disregulation of expression of different cell surface antigens in the leukemic cells. During the process of leukemogenesis, mutational or other events may occur in which some antigens are deleted or others are incorrectly expressed.[37,38] Such changes would splay the extrapolation from leukemic cells to normal counterparts.

The data presented in this article illustrate that multiparameter flow cytometry can be used to identify a single lineage of cells as distinct from all others in a heterogeneous mixture. By comparing the antigens expressed on only these cells, the composition of that defined population can be assessed. These techniques can be extended to examine the sequential acquisition of antigens in other lineages. In addition, the multiparameter approach can be used to identify populations of cells with abnormal cell surface and physical characteristics that may be indicative of particular disease states or hematopoietic abnormalities.

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Therapy of Severe Aplastic Anemia in Young Adults and Children With Allogeneic Bone Marrow Transplantation

By P.B. McGlave, R. Haake, W. Miller, T. Kim, J. Kersey, and N.K.C. Ramsay

During an 8-year period, 28 young adults (median age 27 years) and 30 children (median age 10 years) with severe aplastic anemia have received allogeneic bone marrow transplantation (BMT) from major histocompatibility loci-matched sibling donors after preparation with cyclophosphamide and total lymphoid irradiation (TLI). All recipients were previously transfused. Comparison of post-bone marrow transplantation events in adults and children reveals equivalent median time to engraftment, median duration of hospitalization, median Karnofsky assessment of activity, and equivalent low rejection rate. Although the incidence of moderate and severe acute graft-*v*-host disease (GVHD) and of extensive chronic GVHD was greater in adults than in children, the projected survival at 4 years of adults (67%; 95% confidence interval [CI] 49% to 85%) and of children (73%; 95% CI 57% to 89%) was equivalent. All survivors are transfusion-free and have normal peripheral blood counts. One of 28 adults and 2 of 30 children have experienced rejection, and 1 of these patients survives after a second transplant. No malignancies have been identified following transplantation. An unexpectedly high incidence of hypothyroidism has been detected and may be attributable to preparation of recipients with TLI. Therapy of severe aplastic anemia with allogeneic BMT after preparation with cyclophosphamide and TLI offers a high rate of transfusion-free survival and a low rejection rate in previously transfused young adults and children.

TRANSPLANTATION of bone marrow from HLA-identical siblings has been used successfully to treat severe aplastic anemia. Rejection rates of <10% are common in untransfused recipients.[1,2] Early reports suggested that blood transfusion prior to transplantation resulted in a significant incidence of graft failure and an adverse effect on long-term survival.[3,4] Modifications of the preparative regimens have decreased the rejection rate in this transfused population.[5-8] Improvements in transfusion practices may also have contributed to the reduction in incidence of rejection.[9]

Total lymphoid irradiation (TLI) is a potent form of immunosuppression both in animal models[10,11] and in humans.[12] We have used the combination of TLI and high-dose cyclophosphamide to prepare previously transfused aplastic anemia patients for allogeneic BMT since 1977.[13] In this report, we update our experience with this approach and compare results in children and in young adults.

PATIENTS AND METHODS

Since November 1977, 28 young adults (median age 27 years, range 18 to 45 years) and 30 children (median age 10 years, range 2 to 17.6 years) received BMT as therapy for severe aplastic anemia. Pretransplant recipient and donor characteristics are presented in Table 1. All patients met established criteria for the diagnosis of severe aplastic anemia.[14] All patients were transfused, and all but one had received more than five transfusions, ie, had been exposed to products from more than five separate blood donors prior to BMT. In addition to significant differences in donor and recipient age, adults and children differed in previous transfusion history (<20 *v* >20 transfusions) and in marrow cell dose. All patients or their legal guardians signed informed consent approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota.

Patients were prepared for transplantation with a regimen designed to minimize rejection[12] including cyclophosphamide, 50 mg/kg/day intravenously (IV) (day −6 to day −3), and TLI, 750 cGy midline dose at the umbilicus (day −1). TLI was given in a single dose at a rate of 26 cGy/min with a 4-MV linear accelerator. Patients then received marrow from major histocompatibility (MHC)-matched sibling donors (day 0). All patients received graft-*v*-host disease (GVHD) prophylaxis with methotrexate.[15] Twenty-nine patients received additional GVHD prophylaxis with equine antithymocyte globulin (ATG) and prednisone.[16] Four patients received 5 mg/day of OKT3 given IV from day +8 to day +20 in addition to methotrexate and prednisone.[17]

Acute GVHD organ involvement was graded I through IV.[15] Chronic GVHD was scored as limited or extensive.[18] The term "advanced GVHD" was applied to patients who developed either grade II or greater acute GVHD or extensive chronic GVHD. Mild cutaneous acute GVHD and limited chronic GVHD were treated with topical steroid cream. Advanced GVHD was treated with regimens containing systemic steroids and, in some cases, adjunctive ATG, cyclosporine, or azathioprine therapy.

Statistical methods. Clinical and laboratory data were retrieved from the University of Minnesota Bone Marrow Transplant Database, which contains systematically and prospectively collected data for all BMT recipients and donors. Univariate analyses were performed using the Kaplan-Meier product-limit method to assess the effects of various factors on survival.[19] The Mantel-Cox statistic was used to compare the difference in survival between groups. The sole exception involved assessment of the possible influence of GVHD on survival. Because GVHD is not a baseline patient characteristic and occurs some time after transplant, it was treated as a time-dependent covariate in a Cox proportional-hazards regression analysis.[20] Factors that were significantly associated with survival in univariate analysis were then entered into a Cox stepwise multivariate regression analysis to determine their independent contributions to survival.

RESULTS

Projected survival at 4 years for the adult group is 67% (95% CI 49% to 85%), whereas that of the children is 73%

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Table 1. Pretransplant Donor and Recipient Characteristics

Characteristic	Adults	Children	Significance
n	28	30	NS
Interval from diagnosis to BMT	47 days (26–3,620)	44 days (19–2,829)	NS
Transfusion history			
<20 transfusions	6	14	$P = .06$
>20 transfusions	22	16	
Recipient age			
Median (range)	27 yr (18–45)	10 yr (2–17.6)	$P = .0001$
Donor age			
Median (range)	25 yr (3–50)	10 yr (1–22)	$P = .0001$
Recipient sex			
M	16	16	NS
F	12	14	
Donor sex			
M	16	11	NS
F	12	19	
Marrow cell dose × 10^8/kg of recipient body weight			
Median	3.2	3.8	$P = .05$

Table 2. Posttransplant Recipient Characteristics

Characteristic	Adults	Children
BMT to engraftment		
Median (range)	23 days (15–43)	23.5 days (16–46)
BMT to discharge		
Median (range)	36 days (21–133)	35 days (21–104)
Acute GVHD*		
None	11	22
Grade I	3	1
Grade II	6	2
Grade III	6	4
Grade IV	2	1
Chronic GVHD†		
None	9	18
Limited	1	4
Extensive	12	3
Karnofsky assessment		
Median (range)	100% (60–100)	100% (80–100)
Rejection rate	1/28	2/30
Survival	19/28	22/30

*Two adults and three children died without GVHD at <100 days and are censored at time of death.

†Six adults and five children died before day 100 and are censored at time of death.

(95% CI 57% to 89%) ($P = .58$) (Fig 1). The latest death in the series occurred at 11 months following BMT. All survivors are transfusion independent. Characteristics of post-BMT of the two groups (Table 2) including median times to engraftment, median durations of hospitalization, median Karnofsky assessments of activity in survivors, and rejection rates are equivalent.

GVHD. The incidence and severity of GVHD in adults and in children is shown in Table 2. Kaplan-Meier analysis revealed a higher incidence of moderate and severe acute GVHD at 100 days in adults (54%; 95% CI 35% to 73%) than in children (24%; 95% CI 9% to 40%) ($P = .08$), and a significantly higher long-term projected incidence of extensive chronic GVHD in adults (56%; 95% CI 35% to 78%) than in children (12%; 95% CI = 0% to 25%) ($P = .008$). The long-term projected incidence of advanced GVHD in adults of 68% (95%; CI 49% to 86%) was significantly greater than the 36% projected incidence (95%; CI 18% to 54%) seen in children ($P = .02$) (Fig 2). Twelve patients in this series have required systemic steroid therapy and, in some cases, adjunctive therapy with azathioprine for extensive chronic GVHD. Three of these 12 patients have died, whereas 8 patients have experienced complete resolution of chronic GVHD and are no longer receiving immunosuppressive therapy. One patient is receiving low doses of azathioprine as therapy for persistent cutaneous chronic GVHD.

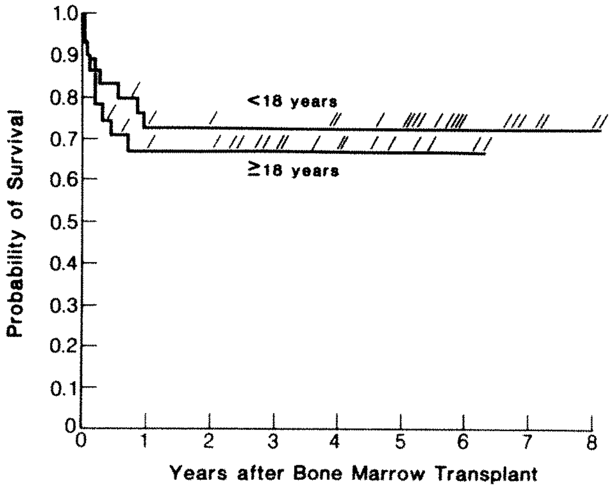

Fig 1. Projected long-term survival of 30 children (<18 years) and 28 adults (18 to 44 years) undergoing BMT for SAA. One surviving child who experienced marrow failure and was retransplanted is a transfusion-free survivor at +78 months.

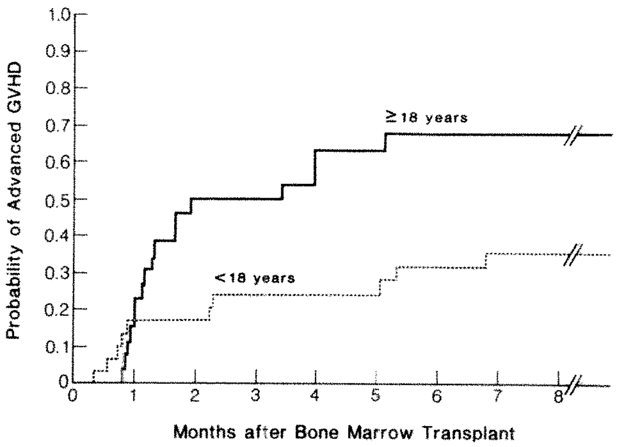

Fig 2. Projected incidence of advanced GVHD in 28 adults and 30 children undergoing allogeneic BMT for SAA. The term "advanced GVHD" defines patients who developed either moderate or severe acute GVHD or extensive chronic GVHD. Last event on either curve is at 22 months. Dead patients have been censored.

Graft failure. Three of 58 patients experienced nonengraftment or rejection following BMT. A 9-year-old girl (UPN 96) with idiopathic severe aplastic anemia who had received <20 transfusions prior to transplantation failed to engraft following an infusion of 3.9×10^8 nucleated bone marrow cells/kg of recipient weight from her MHC-matched brother. A second infusion of 4.4×10^8 cells per cells per kilogram of recipient weight from the same donor following preparation with cyclophosphamide, procarbazine, and ATG was unsuccessful, as was a third infusion of 1.7×10^8 cells/kg of recipient weight. The patient died at +99 days with *Escherichia coli* sepsis and *Aspergillus sp* pneumonia. No evidence of engraftment could be found at time of death.

A 7-year-old girl (UPN 103) with idiopathic severe aplastic anemia who had received <20 transfusions prior to transplantation had successful engraftment at day +25 after receiving 3.9×10^8 cells/kg of recipient weight from her MHC-matched sister. Twenty-nine months later, the patient returned with recurrent severe aplastic anemia. After preparation with cyclophosphamide, 50 mg/kg/day (day −5 to day −2) and TBI 300 cGy (day −1), the patient received 3×10^8 bone marrow cells/kg of recipient weight from the same donor and engrafted at day +19. She is now alive and well at +45 months following a second BMT.

A 26-year-old man (UPN 410) with idiopathic severe aplastic anemia who had received >20 transfusions prior to BMT as well as an unsuccessful course of equine ATG (Minnesota) failed to establish a graft following preparation with cyclophosphamide and TLI, and an infusion of 3.2×10^8 cells/kg of recipient weight donated by his MHC-matched brother. Peripheral blood evidence of engraftment occurred on day +19 and was confirmed by bone marrow biopsy. Aplasia recurred at day +45 and was treated by infusion of 3.1×10^8 cells/kg of recipient weight using bone marrow cells from the same donor following preparation with cyclophosphamide, cytosine arabinoside, and TBI. Engraftment did not occur, and the patient died at day +120 with *Aspergillus sp* pneumonia.

Thyroid dysfunction. "Compensated hypothyroidism," defined as elevation of serum thyroid stimulating hormone (TSH) serum levels >10 μIU/ml on at least one occasion or between 7 to 9.9 μIU/ml on at least two occasions in the presence of a normal T4 index, occurred in 13 of 37 recipients studied at >2 years after BMT. "Primary hypothyroidism," defined as concomitant depression of the serum T4 index (<5) and the above-defined serum TSH elevation, occurred in an additional three recipients.

Pneumonia. In this series, pneumonia occurred in 31 of 58 patients. Etiologies included cytomegalovirus (CMV) (5 cases), fungus (3 cases), bacterial infection (3 cases), *Pneumocystis carinii* (3 cases), acute respiratory distress syndrome (ARDS) (1 case), and pulmonary hemorrhage (1 case). In 15 cases, no etiology was determined.

Cause of death. Seventeen of the 58 patients studied have died. Primary causes of death are listed in Table 3. In 9 of the 17 cases, pneumonia was considered the principal clinical event responsible for death. In one case (UPN 129) rapid onset of fatal pulmonary edema occurred in a patient receiving concomitant amphotericin B empiric therapy and WBC infusion. In a second case (UPN 525), death was related to uncontrollable hemorrhage into the lung after ear, nose, and throat (ENT) surgery. In the remaining 7 cases fatal pneumonia could be attributed to CMV (5 cases), *Candida sp* (1 case), or a bacterial pathogen (1 case). In 4 cases, sepsis with either bacterial pathogens (3 cases) or with CMV (1 case) accounted for death. Meningitis from *Staphylococcus sp* and from *E coli*, respectively, occurring in patients with advanced GVHD, accounted for two additional deaths related to infection. Finally, two patients previously described (UPN 96, UPN 410) died of infectious complications associated with graft failure and prolonged pancytopenia. Advanced GVHD was considered a contributing feature in 11 of the 17 deaths.

Table 3. Cause of Death

Principal Cause*	(GVHD)†	n
Pneumonia		
CMV	5	5
Candida sp		1
Bacterial	1	1
ARDS		1
Hemorrhage		1
Sepsis	3	4
Meningitis	2	2
Graft failure		2
Total	11	17

*Single clinical feature considered most important in patient death.

†GVHD or GVHD therapy considered a contributing factor in patient death.

Survival. Univariate Kaplan-Meier analyses were performed to assess the influence of patient characteristics on survival. No significant correlation with survival could be found when recipient age or sex, donor age or sex, recipient/donor sex match, or GVHD prophylaxis were considered. Prolonged interval from diagnosis to BMT defined as greater than the median interval of 46 days ($P = .01$) and an extensive pretransplant transfusion history (>20 U) ($P = .02$) predicted decreased survival. The occurrence of advanced GVHD was treated as a time-dependent covariate in a Cox regression analysis of survival. Those with advanced GVHD had significantly decreased survival ($P = .0004$). Survival was also analyzed by decade of recipient age (Table 4). Although patients aged >30 years had a trend toward poorer survival, there was no significant difference in survival among the four age groups ($P = .75$).

A Cox multivariate analysis was then conducted to assess the independent contribution of factors that were significant

Table 4. Projected Long-term Survival by Decade

Age	Number	Survival (%)	95% CI
0-9	16	75	54-96
10-19	20	75	55-94
20-29	12	67	40-93
30-44	10	58	27-90

Table 4 depicts the projected long-term survival of BMT recipients analyzed by age. There is no significant difference in projected survival among the four groups ($P = .75$)

in univariate analyses. Because the effect of recipient age on outcome was of interest, it was included in the analysis and was forced into the model at the first step. The results of this analysis are presented in Table 5. Advanced GVHD (P = .002) and a prolonged interval from diagnosis to transplant (P = .01) predicted poorer outcome. Neither recipient age nor previous transfusion history significantly influenced survival when occurrence of advanced GVHD and interval from diagnosis to BMT were taken into account.

DISCUSSION

In this report, we demonstrate that transplantation of bone marrow taken from MHC-matched sibling donors provides excellent survival for previously transfused children and young adults with severe aplastic anemia. Preparation of recipients with high-dose cyclophosphamide and TLI is associated with a low rejection rate (3 of 58) in a group considered at high risk for marrow failure and has not been associated with occurrence of malignancy. Prolonged interval from diagnosis to transplantation and development of advanced GVHD independently predict poor outcome. The flat, transfusion-free survival curve from 11 months to 9 years (Fig 1) reflects the virtual absence of life-threatening complications occurring in patients surviving the acute posttransplant course.

A graft failure rate of <10% has been consistently reported when untransfused patients receive BMT therapy for severe aplastic anemia.[1,2] Early reports suggested that transplantation of previously transfused patients resulted in a significant incidence of graft failure and an adverse effect on long-term survival.[3,4] Graft failure was attributed to nonspecific sensitization of the recipient by antigens found in transfused blood products.[21,22]

Subsequently, modifications of the preparative regimen have been designed to reduce the rate of graft failure in transfused recipients. Addition of nonirradiated donor peripheral blood buffy coat following marrow infusion has reduced the incidence of graft failure from 32% to 14%.[5] Infusion of buffy coat, however, is associated with an increased incidence of chronic GVHD.[23]

Immunosuppression with a combination of total body irradiation (300 cGy) given in a single dose and high-dose cyclophosphamide resulted in a projected survival rate of 61% and rejection in only 1 of 44 previously transfused severe aplastic anemia patients. A high incidence of interstitial pneumonitis and a high mortality in adults, however, was reported.[6] Addition of cyclosporine to the BMT preparative regimen has been reported to reduce the risk of early graft failure.[7] A subsequent report from the same authors, however, demonstrated that unexpected late graft failures may occur after withdrawal of cyclosporine therapy.[24] When immunosuppression with cyclosporine is combined with high-dose cyclophosphamide, both early graft failure and fatal venoocclusive disease of the liver can occur.[25] Preparative immunosuppression with a combination of procarbazine, rabbit antithymocyte serum (ATS), and high-dose cyclophosphamide has also been reported to provide a low rejection rate (10%) and acceptable projected long-term survival (61%) in a group of relatively young recipients (median age 17 years).[8]

Table 5. Multivariate Analysis of Characteristics Predicting Poor Outcome

Characteristic	Relative Risk	Significance (p Value)
Advanced GVHD	5.9	.002
Prolonged interval from diagnosis to BMT	2.4	.01
History of >20 transfusions prior to BMT	—	.10
Age >18 years	—	.58

Table 5 depicts results of stepwise multivariate analysis correlating various risk features with death in 58 transfused patients undergoing BMT for SAA.

The 5% rejection rate in this series compares favorably with rejection rates reported after BMT therapy of either untransfused or previously transfused aplastic anemia patients. This low rejection rate is probably the result of preparative immunosuppression with TLI, which was first shown to be immunosuppressive in animal models.[10,11] Other researchers have reported preliminary results suggesting a low rejection rate after preparation of transfused aplastic patients with low-dose TLI.[26] Radiation ports used to deliver TLI include portions of the thyroid gland. In this series, 16 of 37 recipients studied at >2 years following transplantation had chemical evidence of hypothyroidism.[27,28] Endocrine abnormalities occurring after preparation with single-dose TBI and allogeneic BMT have been reported.[29] Hypothyroidism occurring after mantle irradiation therapy of Hodgkin's disease is also common, often clinically insignificant, and sometimes reversible without therapy.[30,31] TLI may have similar adverse effects when used for bone marrow transplantation therapy of severe aplastic anemia.

Pneumonia was a principal clinical feature in 9 of the 17 deaths in this series. The contribution of irradiation to the development of fatal pneumonia is difficult to determine. Damage to the pulmonary bed itself is unlikely since individually tailored lung shielding permitted <10% of the total calculated dose of irradiation to penetrate the center of the lung block in all recipients. In seven of the nine cases of fatal pneumonia, an infectious pathogen was demonstrated. The additive immunosuppressive effect of TLI and cyclophosphamide may have predisposed recipients to pneumonia in these cases. Most recipients with fatal pneumonia, however, also were receiving treatment for advanced GVHD. GVHD and therapy for GVHD have been associated with the development of pneumonia following allogeneic BMT for leukemia.[32] The relative importance of these etiologic features on the development of fatal infectious pneumonia is not known.

The long-term, projected incidence of advanced GVHD was 67% in adults and 36% in children, and advanced GVHD contributed significantly to mortality in this series. The reason for the disparity between the projected incidence of GVHD in adults and in children is not obvious. An increased incidence of GVHD has occurred in severe aplastic anemia patients receiving nonirradiated donor buffy coat infusions.[23] Adults in this series, however, received a lower median cell dose per kilogram of recipient weight (3.2×10^8) than did children (3.8×10^8) (P = .05), and neither children nor adults received donor buffy coat infusions. The relative importance of older donor age in the development of GVHD is difficult to assess in this series since donor age parallels recipient age in both the older and in the younger group. Despite the increased incidence of advanced GVHD in adults, no significant disparity in parameters associated with outcome (including time to engraftment, rejection rate, median length of hospitalization, or median Karnofsky assessment of survivors) could be found. Recent reports suggest that prophylaxis with the combination of methotrexate and cyclosporine after allogeneic BMT therapy for severe aplastic anemia can result in an extremely low cumulative incidence of moderate or severe acute GVHD (14%) with a low rejection rate and with excellent projected survival.[33] Studies that compare the relative merits of our current acute GVHD prophylaxis to prophylaxis with the combination of methotrexate, cyclosporine, and prednisone are underway at our institution.

In this study, excellent long-term transfusion free survival occurred in both young adults (18 to 44 years) and in children (1 to 17 years). Comparison of survival by decade demonstrated no significant difference, although only 10 adult recipients were between the ages of 30 and 44 years. An inverse correlation of survival and increasing recipient age following BMT for severe aplastic anemia has been reported[6]; however, the comparable survival in children and young adults in our series is in agreement with two recently reported analyses.[34,35]

The observation has been made that patients receiving allogeneic BMT therapy for severe aplastic anemia in the "modern era" have lower rejection rates and better survival than patients transplanted with similar preparative regimens in earlier years. Low rejection rates, comparable to those that are observed after transplant of untransfused severe aplastic anemia patients, have been attributed to a decreased incidence of recipient sensitization accompanying improvements in blood product transfusion procedures.[9] Better overall survival may be attributable to expedited therapy with BMT, and better pretransplant and posttransplant support.[8] The results of our study are in agreement with reports of better survival in recently transplanted patients. In our experience, projected long-term transfusion-free survival of 28 patients (median age 14 years) transplanted from 1977 to 1981 is 61% (95% CI 43% to 79%), whereas projected long-term survival of 30 patients transplanted from 1982 to 1986 (median age 21 years) is 79% (95% CI 64% to 94%) ($P = .10$).

Allogeneic bone marrow transplantation therapy for severe aplastic anemia after preparation with a combination of cyclophosphamide and TLI provides excellent transfusion-free survival and an extremely low rate of graft failure in young adults and in children. Results of this approach compare favorably with those that have been observed after transplantation with other reported preparative regimens or after therapy with alternative forms of immunosuppression.

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Mixed Hematopoietic Chimerism Following Bone Marrow Transplantation for Hematologic Malignancies

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Twenty-nine of 172 patients (17%) who received an allogeneic bone marrow transplant (BMT) from histocompatible sibling donors for hematologic malignancies were mixed hematopoietic chimeras; ie, they had a mixture of donor and host hematopoietic or lymphohematopoietic cells at ≥14 days after transplantation. Twenty-four of the 29 mixed chimeras (83%) have remained in continuous complete remission for up to 116 months (>9 years) following BMT. Four of the 29 patients (14%) have had recurrent leukemia, and 7 of the 29 (24%) have had moderate or severe graft-*v*-host disease (GVHD). Twelve of these 29 patients have persisted as stable mixed chimeras for ≥2 years after BMT, whereas other patients converted to all donor-type hematopoiesis. The incidence of mixed chimerism was independent of the pretransplant regimen, the donor or recipient age (<20 *v* >20 years), remission status (first complete remission of acute leukemia and first chronic phase of chronic myelocytic leukemia *v* later stages of disease), and type of leukemia. Our data indicate that mixed hematopoietic chimerism is not rare after BMT for hematologic malignancies and that its presence is compatible with long-term disease-free survival. Prospective studies of mixed chimerism after BMT are warranted to achieve better understanding of its biologic importance.

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WE PREVIOUSLY reported nine patients treated with high-dose radiochemotherapy and allogeneic bone marrow transplantation (BMT) for acute leukemia who became mixed hematopoietic chimeras; that is, they had blood cells of both donor and recipient origin.[1,2] We have subsequently carried out studies on 172 patients treated with radiochemotherapy and BMT for hematologic malignancies to characterize mixed chimerism in detail, to document its incidence using two different preparatory regimens, and to determine its relationship to graft-*v*-host disease (GVHD), leukemic relapse, and disease-free survival. We also evaluated the advantages and disadvantages of several genetic markers for such studies, including the use of DNA restriction fragment length polymorphisms (RFLPs).[3]

PATIENTS AND METHODS

Patient Population

We evaluated 293 patients who received an allogeneic bone marrow transplant for leukemia at the City of Hope National Medical Center between May 18, 1976 and December 31, 1985. Patients with a unique patient number (UPN) of 1 to 281 were evaluated retrospectively, whereas patients with a UPN of 282 to 336 were studied prospectively. We considered only patients who had one or more informative genetic markers tested after BMT to determine the presence or absence of host-type hematopoietic cells. Detailed information is available in 172 patients at this time. The diagnoses were as follows: acute lymphocytic leukemia (ALL), n = 65; acute nonlymphocytic leukemia (ANLL), n = 72; chronic myelocytic leukemia (CML), n = 32; refractory anemia with excess blasts, n = 1; chronic myelomonocytic leukemia (CMMOL), n = 1; and hairy cell leukemia, n = 1. All patients received a marrow transplant from histocompatible sibling donors.

Preparatory Regimens

Patients with a UPN of 1 to 185 were treated with a pretransplant preparative regimen (regimen 1) consisting of cytosine arabinoside (Ara-C), high-dose cyclophosphamide, and 1,000 cGy total body irradiation (TBI) delivered at 7 cGy/min,[4] except for patients 78 and 100 who received 750 cGy of TBI at a dose rate of 26 cGy/min. The preparative regimen for patients 186 to 334 (regimen 2) consisted of 1,320 cGy TBI given in 11 fractions of 120 cGy,[5] and cyclophosphamide, VM 26, or VP 16.[6]

The posttransplant immunosuppressive regimen for patients 1 to 177 consisted of methotrexate and prednisone,[4] whereas subsequent patients were randomized to receive either methotrexate and prednisone or cyclosporine and prednisone.[7]

Genetic Markers

The following genetic markers were used: RBC antigens, sex chromosomes, immunoglobulin allotypes, and DNA restriction fragment length polymorphisms (RFLPs).

RBC antigens and antibodies. RBC phenotyping was performed using standard techniques.[8] Typing for the following antigens was performed: A_1, B, A, D, C, E, c, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, P_1, M, N, S, and s. Transfusions prior to referral for transplantation occasionally made the determination of the patient's RBC phenotype difficult. In those instances, reticulocyte enrichment of the patient's RBCs[9] aided in establishing the correct phenotype. It was frequently also impossible to exclude mixed chimerism by RBC antigen typing during the first 3 to 5 months after transplantation because of transfusions. In ten patients, the presence of donor-type RBC antigens after BMT in the absence of mixed field reactions was

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considered an indication of complete allogeneic marrow engraftment.

We interpreted an RBC alloantibody or an autoantibody in a patient prior to BMT that remained detectable for >6 months after BMT as an indication of the persistence of host B lymphocytes. This occurred most commonly in transplants involving a major ABO blood group incompatibility (eg, donor group A, patient group O), which was present in 11 patients (UPN 38, 47, 78, 95, 108, 118, 168, 233, 268, 279, and 320). We selected the 6-month period as being well beyond the expected duration of persistence of antibody since previous studies have indicated that patients' RBC alloantibodies ordinarily persist for no longer than 120 days after BMT.[10]

Cytogenetic studies. In patients who received marrow from a donor of the opposite sex, cytogenetic analyses were performed,[11] usually using bone marrow specimens. In some instances, peripheral blood lymphocytes were used instead, in which case the lymphocytes were cultured in a conditioned medium described by Mills et al.[12] This medium activates a wide variety of T and B cells as indicated by studies of T and B cell neoplasms in our laboratory (A.D. Stock et al, unpublished observations). The presence of the Y chromosome was detected by screening metaphases after quinicrine staining or using C-banding. When adequate numbers of metaphases were available, at least 30 were so screened.

Immunoglobulin allotyping. Immunoglobulin allotyping was performed using hemagglutination inhibition. The method and the reagents used were as previously reported.[13,14] To exclude the presence of exogenous immunoglobulins as a result of blood transfusion, allotyping was performed at ≥100 days after the most recent transfusion.

DNA RFLP analysis. Restriction fragment analysis was performed as previously described.[3] In brief, high-mol-wt DNA was isolated from the nuclei of peripheral blood cells obtained from ~25 mL blood collected in heparin or in 10% acid-citrate-dextrose. The purified DNA was digested with restriction endonucleases; the DNA fragments were then fractionated by electrophoresis in 0.5% to 1.0% agarose gels, and hybridization was carried out using the appropriate probes either in gel or after Southern blotting[15] using nitrocellulose (Schleicher and Schuell, Keene, NH), Genetran (Plasco, Woburn, MA), or Zeta-bind (AMF Cuno, Meriden, CT) filters.

A panel of ten DNA probes was available for hybridization.[3] These included pAW101[16] (obtained from Dr Ray White, Salt Lake City), pDP34[17-19] (obtained from Dr David C. Page, Boston), AT3[20] (obtained from Dr David Ginsburg, Ann Arbor, MI), Gγ and Aγ globin gene oligonucleotide probes (prepared by one of us, RBW), and five randomly cloned single copy DNA sequences termed LAM4-123, -1065, -427, -355, and -1214[21] prepared and characterized at Collaborative Research, Inc (Lexington, MA). The genomic probes were labeled by nick translation with ^{32}P-deoxynucleotide triphosphate (New England Nuclear, Boston) to a specific activity of 1 to 10 × 10^8 CPM/μg DNA; 5' end labeling of oligonucleotide probes was performed using T4 polynucleotide kinase.[22]

Definitions

We defined a patient as a "complete chimera" when genetic markers detected only donor-type hematopoietic cells after BMT.[23] We defined a transplant recipient as a "mixed hematopoietic chimera" or "mixed chimera" if the patient had a mixture of donor and host hematopoietic or lymphohematopoietic cells ≥14 days after BMT. We have excluded those patients who were complete chimeras and subsequently had a reemergence of host cells only at or near the time of relapse of their leukemia. In this regard, we excluded ten patients who developed hematologic evidence of relapse within 90 days of the reemergence of detectable host cells.

RESULTS

Incidence of Mixed Hematopoietic Chimerism

One of the aims of our study was to determine the incidence of mixed hematopoietic chimeras following BMT and, in particular, to determine if there was a difference in incidence in patients treated with 1,000 cGy single-dose TBI (regimen 1) as compared with patients treated with 1,320 cGy fractionated TBI (regimen 2). Twenty-nine of the 172 patients studied (17%) were mixed hematopoietic chimeras. Eighteen of 81 patients (22%) treated with regimen 1 and 11 of 91 patients (12%) treated with regimen 2 became mixed chimeras. This difference in incidence is not statistically significant (P = .10). The clinical characteristics of the mixed hematopoietic chimeras are presented in Tables 1 and 2. Mixed chimeras have been documented among patients with various types of leukemia; ie, 12 of the patients had ALL, 13 had ANLL, 2 had CML, 1 had chronic myelomonocytic leukemia, and 1 had hairy cell leukemia.

Frequency of Informative Markers and Sensitivity of the Various Genetic Markers

RBC antigen phenotyping prior to BMT in 275 donor/recipient pairs revealed donor and recipient markers in 201 pairs (73%), only recipient-specific markers in 37 pairs (13%), only donor-specific markers in 29 pairs (11%), and no markers in 8 pairs (3%). A minor population of RBCs can generally be detected by RBC phenotyping when their percentage is as low as 1% to 5% of the total.

Immunoglobulin allotype testing in 75 donor/recipient pairs revealed a recipient-specific marker or both donor-specific and recipient-specific markers in 37 pairs (49%), donor-specific markers in 12 pairs (16%), and no markers in 26 pairs (35%). Immunoglobulin allotypes can be detected to a level of ~3% of normal.

DNA RFLP can be informative in essentially 100% of donor/recipient pairs depending on the number of probes used for hybridization and their polymorphism information content.[3,21,24,25] Using probes pAW101, pDP34, AT3, Gγ globin, and Aγ globin, a recipient-specific marker or both recipient-specific and donor-specific markers were present in 38 of 40 (95%) donor/recipient pairs tested. With the five LAM4 randomly cloned genomic probes,[3,21] the observed frequency of finding a recipient-specific marker in 30 sibling pairs was 100%. DNA RFLP can detect ~5% to 10% of a minor population of donor or host DNA.[3,21,25,26]

The sensitivity of sex chromosome analysis for detection of host cells depends on the number of cells examined. For example, one must examine ≥59 cells to exclude 5% host cells with 95% confidence.[27]

Results of Genetic Marker Studies

The results of the genetic marker studies used to define the patients as being mixed hematopoietic chimeras are shown in Tables 3 and 4. As indicated, some of the patients have remained stable mixed chimeras for extended periods of time (eg, UPN 78, 100, and 144) whereas others eventually

Table 1. Clinical Characteristics of Mixed Hematopoietic Chimeras

	Patients Transplanted in First Complete Remission									
UPN	Diagnosis	Remission Status	Age at BMT	Prep Regimen	D/R Sex	GVHD Prophylaxis	Acute GVHD	Chronic GVHD	Outcome of BMT	Karnofsky Score
47	ANLL	1st CR	28	1	F/F	MTX/PSE	II	None	CCR +2,638	100
64	ALL	1st CR	21	1	M/M	MTX/PSE	I	None	CCR +2,457	100
78	ALL	1st CR	2	1	M/F	MTX/PSE	0	None	CCR +2,317	100
95	ANLL	1st CR	27	1	M/F	MTX/PSE	0	None	CCR +2,163	100
101	ANLL	1st CR	20	1	F/M	MTX/PSE	I	None	CCR +2,093	80
108	ANLL	1st CR	18	1	M/M	MTX/PSE	I	None	Relapsed +553; died +1,059	0
118	ANLL	1st CR	16	1	M/M	MTX/PSE	III	None	CCR +1,946	100
152	ALL	1st CR	23	1	F/M	MTX/PSE	II	None	CCR +1,617	100
160	ANLL	1st CR	35	1	F/M	MTX/PSE	0	Moderate; resolved	CCR +1,561	90
168	ANLL	1st CR	24	1	M/M	MTX/PSE	0	None	CCR +1,491	100
179	ANLL	1st CR	20	1	M/F	CSA/PSE	I	None	CCR +1,372	100
268	CML	1st CR	38	2,CY	M/F	CSA/PSE	I	None	CCR +714	100
279	ALL	1st CR	30	2,CY	M/F	CSA/PSE	0	None	CCR +637	90
317	ANLL	1st CR	18	2,CY	M/F	MTX/PSE	0	None	CCR +420	100
320	ALL	1st CR	23	2,CY	M/M	CSA/PSE	0	None	CCR +399	100
323	ALL	1st CR	28	2,CY	F/M	CSA/PSE	0	None	CCR +378	100

Abbreviations: CCR, continuous complete remission; MTX, methotrexate; PSE, prednisone; CSA, cyclosporine; CY, cyclophosphamide.
Date of evaluation (Tables 1 and 2): November 17, 1986.

converted to all donor-type hematopoiesis (eg, UPN 233 and 283).

The percentages of host cells in mixed chimeras varied from patient to patient and were most readily determined from chromosome analysis when adequate numbers of metaphases were available. Of the 11 patients determined to be mixed chimeras by chromosome analysis, ≥30 metaphases were examined in 9 patients; in the other 2 patients, 80 and 20 metaphases were examined, respectively. Six of the 11 patients had >50% host cells, and the other 5 patients had 2.5%, 5%, 10%, 15%, and 28% cells of host origin. Repeat studies revealed a similar percentage or a decreasing percentage of host cells. Bone marrow was the source of the specimens in 8 of the 11 patients, peripheral blood was the source in 1, and both marrow and blood were the sources in 2.

The percentage of host cells on initial diagnosis of patients as mixed chimeras did not predict whether the patient would be a stable mixed chimera or would convert to a complete chimera. For example, of those mixed chimeras who converted to all donor-type cells, the initial proportion of host-type cells was only 2% and 10% in patients 38 and 260, respectively, but was 87% and 90% in patients 233 and 234.

The percentages of host and donor erythrocytes were estimated by observance of the mixed field reactions using RBC typing reagents. RBCs were largely of host origin in

Table 2. Clinical Characteristics of Mixed Hematopoietic Chimeras

	Patients Transplanted Not in First Complete Remission									
UPN	Diagnosis	Remission Status	Age at BMT	Prep Regimen	D/R Sex	GVHD Prophylaxis	Acute GVHD	Chronic GVHD	Outcome of BMT	Karnofsky Score
9	ALL	2nd REL	29	1	F/F	MTX/PSE	0	None	CCR +3,542	100
38	ALL	2nd REL	17	1	M/F	MTX/PSE	I	None	Relapsed +365; died +395	0
100	ANLL	1st REL	2	1	M/F	MTX/PSE	0	None	CCR +2,100	100
107	ALL	2nd CR	28	1	M/M	MTX/PSE	0	None	CCR +2,037	100
144	ANLL	IF	25	1	F/M	MTX/PSE	0	Mild; resolved	CCR +1,673	100
163	ALL	2nd REL	8	1,VM26	M/M	MTX/PSE	0	None	Relapse/died +896	0
171	ALL	3rd CR	20	1	F/M	MTX/PSE	III	Severe	Died +502; pneumonia	0
222	ANLL	IF	29	2,VP16	F/F	MTX/PSE	III	Moderate; resolved	CCR +1,092	100
226	HCL	2nd REL	35	2,CY	M/M	CSA/PSE	II	Mild; resolved	CCR +1,001	100
227	ALL	2nd CR	29	2,CY	M/M	MTX/PSE	II	None	CCR +994	100
233	CMMOL	PU	41	2,VP16	M/F	CSA/PSE	0	None	CCR +945	100
234	ANLL	2nd REL	14	2,VP16	F/M	CSA/PSE	II	Moderate; improving	Relapsed +903	70
283	CML	AP	33	2,CY	M/F	MTX/PSE	0	None	CCR +609	90

Abbreviations: IF, induction failure; PU, previously untreated; AP, acute phase.

Table 3. Mixed Hematopoietic Chimeras; Pretransplant Regimen 1

UPN	RBC Antigens	Sex Chromosomes	Ig Allotypes	DNA RFLP	RBC Antibodies
9	NI	NA	Mixed: 24,95	NT	—
38	NT	Complete: 0.5,10 Mixed: 1,3	NT	NT	—
47	NI	NA	NT	NT	Persistent anti-A: 65
64	Mixed: 72	NA	NT	NT	—
78	Mixed: 12,16,18,38,58,62	Mixed: 1,18,38,62	NI	Mixed: 70	—
95	Mixed: 8	NT	NT	NT	—
100	Mixed: 12,25,53	Mixed: 1,12,24,53	NI	Mixed: 53	—
101	Mixed: 49	Complete: 49	NT	NT	—
107	NI	NT	Mixed: 47	Complete: 47	—
108	Mixed: 12	NA	NT	NT	Persistent anti-A: 12
118	Mixed: 10,11	NA	NT	NT	—
144	Mixed: Multiple tests to 49	Mixed: 12,32,34,41	Mixed: 14,20	Mixed: 34,41	—
152	Mixed: 5,13 Complete: 16,20,24,27	Complete: 18,27	Mixed: 12,18,27	NT	—
160	Mixed: 11,14,26,30 Complete: 32	Mixed: 1,26 Complete: 3,12,28,43	Mixed: 10,14,16,43	Complete: 32,43	—
163	Mixed: 17	NA	NI	NT	—
168	Impaired erythropoiesis	NA	Mixed: 9,14	NT	Persistent anti-A: 6
171	NT	Host: 1 Mixed: 3	NT	NT	—
179	Mixed: 20,24,35	Complete: 24	Mixed: 24,35	Complete: 24,35	—

Numbers following Mixed refer to months after BMT at which time mixtures of donor and recipient cells were found (mixed chimeras). Numbers following Complete refer to months after BMT at which time only donor genetic markers were detected. Numbers following Host refer to number of months after BMT at which time only cells of host type were found. NA, not applicable (donor and recipient of same sex); NI, not informative.

patients 78, 100, and 108, whereas they were largely of donor origin in patients 95, 101, 118, 152, 160, 163, 179, 222, and 233. Approximately equal percentages of RBCs of donor and host origin were found in patients 64 and 144.

The percentage of host immunoglobulins was determined in selected patients by titration using doubling dilutions of the patients' sera. Approximately 3% to 6% of host immunoglobulins were present in patients 160, 168, 222, and 226. In patients 152, 160, and 222, immunoglobulin allotypes ultimately became the only indication of the presence of host-type cells, whereas earlier in these patients' courses other genetic markers had also indicated mixed chimerism.

Quantitative studies were not performed using DNA RFLPs. Nevertheless, in patients 78, 100, and 144 a comparison of the intensity of the bands of donor and recipient origin made it evident that ≥50% of the DNA was of host origin, in agreement with the results of other genetic markers.

Correlation of Results of Various Genetic Markers

Cytogenetic studies and RBC phenotyping were performed concurrently in a number of patients. Both methods indicated a predominance of host cells in patients 78 and 100, a predominance of donor cells in patient 160, and approximately equal percentages of host and donor cells in patient

Table 4. Mixed Hematopoietic Chimeras; Pretransplant Regimen 2

UPN	RBC Antigens	Sex Chromosomes	Ig Allotypes	DNA RFLP	RBC Antibodies
222	Mixed: 6,9,11,14 Complete: 26	NA	Mixed: 14,26	NT	—
226	NI	NA	Mixed: 12	NT	Persistent RBC autoantibody: 12
227	Complete: 4,12,24	NA	Mixed: 12,23	NT	—
233	Mixed: 5,11,15 Complete: 22	Mixed: 3 Complete: 5,6,10,12,18	NT	NT	—
234	Complete: 12,13,17,21	Mixed: 3 Complete: 1,12,13,17	NT	NT	—
268	NI	Complete: 2,4,5,6	NI	NT	Persistent anti-B and anti-D: 16
279	Impaired erythropoiesis	Complete: 1,2,4,8	NT	NT	Persistent anti-B: >23
283	NT	Mixed: .75,2 Complete: 1,2.5,4,13	NT	Mixed: 2 Complete: 1,2.5,4	—
317	Complete: 7	Mixed: 2 Complete: 1,4,6	NT	Complete: 1,2,3	—
320	Mixed: 7,9 Complete: 12	NA	NT	Complete: 2,3	persistent anti-B: 8
323	Complete: 5	Mixed: 4 Complete: 1	NT	NT	—

144. In patients 101, 152, and 179, RBC phenotyping indicated a small population of host cells, whereas cytogenetic studies revealed only cells of donor origin. In patients 234 and 323, RBCs were all of donor phenotype, whereas a small percentage of host cells (2.5% and 10%) were detected on a single examination in each patient by chromosome analysis. In patient 233, RBCs were largely of donor origin, and host cells were detected by chromosome analysis in one of six studies.

In patient 283, in vitro mixing experiments using mixtures of DNA of host and donor origin indicated that ~20% of DNA was of host origin at a time when 9 of 60 metaphases (15%) were of patient type.

RBC antibodies persisting >6 months after BMT served as the basis of diagnosing mixed chimerism in 7 patients; in 2 of these patients immunoglobulin allotyping also indicated mixed chimerism, whereas in the other 5 patients immunoglobulin allotyping either was uninformative or was not performed. Anti-A or anti-B antibodies were present in 6 patients and caused a direct antiglobulin test without hemolysis in 2 patients, a positive direct antiglobulin test with transient hemolytic anemia in 1 patient, and a delayed onset of erythropoiesis (170 days to >605 days after BMT) in 3 patients.[28,29] As with other genetic markers, host RBC alloantibodies ultimately disappear in most patients, but patient 279 still has a high titer anti-A antibody. She is also transfusion dependent because of a lack of erythropoiesis by the engrafted marrow >605 days after BMT.[29]

Clinical Outcome

In this largely retrospective study, all marrow transplant patients who were seen in follow-up during the study period were included. An analysis of our data revealed, however, that patients who were complete chimeras were first studied at a median of 3 months posttransplant (range 0.5 to 103 months), whereas those patients who were mixed chimeras were first studied at a median of 6 months posttransplant (range 0.75 to 72 months). A statistical comparison of clinical outcome of mixed chimeras and complete chimeras is therefore not feasible. Nevertheless, it is noteworthy that 24 of the 29 mixed chimeras (83%) have remained in continuous complete remission (CCR) and that only 4 of the 29 mixed chimeras (14%) have had a relapse of their hematologic malignancy. In addition, the incidence of moderate and severe GVHD in this heterogeneous population was 24%.

DISCUSSION

Our data indicate that 29 of 172 (17%) patients treated with marrow ablative high-dose radiochemotherapy and BMT for hematologic malignancies became mixed hematopoietic chimeras. Twelve of these 29 patients were mixed chimeras for ≥2 years after BMT, as documented by one or more genetic markers. Moreover, 24 of the 29 mixed chimeras (83%) have remained in CCR for up to 116 months (>9 years) following BMT.

The existence of mixed hematopoietic chimeras who are in long-term CCR indicates that the mechanism by which remission is produced is not simply that of marrow ablation followed by rescue with donor marrow. A graft-*v*-leukemia effect[30,31] may contribute to the maintenance of remission after BMT, but this effect is not essential since long-term CCR can occur after syngeneic marrow transplantation and after chemotherapy alone. Thus, the mechanism by which high-dose radiochemotherapy and BMT lead to mixed chimerism and long-term remission may be the selective destruction of a leukemic clone while allowing for repopulation of the marrow with normal endogenous hematopoietic cells along with growth of donor cells. Alternatively, since evidence exists for a multistep pathogenesis of some leukemias,[32] therapy in some patients may eradicate a leukemic subclone, resulting in restoration of morphologically and cytogenetically normal marrow even though an abnormal clone of host cells may persist.[32] Indeed, the presence of a clonal population of mature blood cells in patients in complete hematologic remission has been reported.[33,34]

Only 4 of 29 mixed chimeras not in overt hematologic relapse within 90 days of the time of detection of host cells subsequently relapsed. On the other hand, we[3] and other authors[35] have pointed out that a progressive increase in the percentage of host cells is associated with a poor prognosis due to recurrent disease. Thus, when mixed chimerism is diagnosed, follow-up genetic marker studies and hematologic tests are necessary to distinguish relapse from stable mixed chimerism.

Factors that lead to the production of mixed chimerism after high-dose radiochemotherapy in some patients but not others have not been identified. In our patients, the incidence of mixed chimerism was independent of the pretransplant regimen, the donor or recipient age (<20 *v* >20 years), remission status (first complete remission of acute leukemia and first chronic phase of chronic myelocytic leukemia *v* later stages of disease), and type of leukemia.

A major ABO blood group incompatibility was present in 11 of 29 (38%) patients who became mixed chimeras but in only 20 of 143 (14%) patients who became complete chimeras. This difference is statistically significant ($P = .006$). If the three patients whose only genetic marker was an RBC alloantibody are excluded however, the difference is no longer significant. Thus, the higher incidence of mixed chimerism in transplants in which there is a major ABO blood group incompatibility may reflect that a test for RBC alloantibodies is a more sensitive marker than the other genetic markers used. Patients without major ABO incompatibilities are not eligible for such an analysis.

Mixed hematopoietic chimerism has also been reported after BMT by other investigators.[23,25,26,32,35,36-43] Indeed, Hill et al[23] reported the remarkable fact that the incidence of mixed chimerism was 58.3% in their series of 96 patients who received a marrow transplant for aplastic anemia. Mixed chimerism persisted for up to 395 days posttransplant, with the graft then being rejected or, more commonly, with hematopoiesis converting to 100% donor-type cells. The Kaplan-Meier estimate of the probability of developing grades II to IV acute GVHD for mixed chimeras and complete chimeras was significantly different, showing an increase in the frequency of acute GVHD in the complete chimeras. The marrow rejection rate, however, was significantly increased in mixed chimeras as compared with complete chimeras although the projected survival rate was not different.

Prospective studies of mixed hematopoietic chimeras after BMT are warranted for a better understanding of its biologic importance. Moreover, insights into basic aspects of transplantation biology may be possible by analysis of hematopoietic repopulation in the posttransplant period.

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Association of a Chromosomal 3;21 Translocation With the Blast Phase of Chronic Myelogenous Leukemia

By Charles M. Rubin, Richard A. Larson, Mitchell A. Bitter, John J. Carrino, Michelle M. Le Beau, Manuel O. Diaz, and Janet D. Rowley

An identical reciprocal translocation between the long arms of chromosomes 3 and 21 with breakpoints in bands 3q26 and 21q22, t(3;21)(q26;q22), was found in three male patients with the blast phase of chronic myelogenous leukemia (CML). The abnormality was clonal in all three patients and was always accompanied by either a standard or variant 9;22 translocation resulting in a Philadelphia chromosome (Ph^1). In two cases, the t(3;21) was the only abnormality other than a t(9;22) in the primary clone. Serial studies of one patient demonstrated that the t(3;21) occurred as a result of clonal evolution near the time of development of the blast phase. We have not observed the t(3;21) in >500 patients with CML in the chronic phase. Thus, the t(3;21) is a new recurring cytogenetic abnormality associated with the blast phase of CML.

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THE LEUKEMIA CELLS of virtually all patients with chronic myelogenous leukemia (CML) are characterized by the Ph^1 or Philadelphia chromosome, a product of a reciprocal 9;22 translocation, t(9;22)(q34;q11), or of a variant of this translocation.[1,2] The t(9;22) is present at the time of diagnosis of CML and throughout the course of the disease. Evidence is accumulating that the molecular consequence of this rearrangement, namely, fusion of sequences of the *ABL* and *bcr* genes, plays a critical role in the pathogenesis of CML.[3-5]

At diagnosis and during the chronic phase of CML, the t(9;22) is usually the sole cytogenetic abnormality; additional abnormalities are found in 9% to 30% of cases.[6-10] Subsequently, karyotypic evolution occurs in 80% of individuals before or during the blast phase.[9] Numerous secondary chromosomal abnormalities have been observed; however, only a few recurring abnormalities have been identified. The most common of these are +8, +Ph^1, and i(17q); at least one of these is found in 80% of individuals with evolution.[9,11] Rarely, specific abnormalities first identified in acute nonlymphocytic leukemia (ANLL) are noted. These include the t(15;17) of acute promyelocytic leukemia[12,13] and inv(3) and t(3;3) [also interpreted in some reports as an ins(3;3)] of ANLL with abnormal megakaryocytopoiesis[14-19]; in cases of CML with these abnormalities, the acute phase has the characteristic features of the corresponding ANLL.

We identified a new recurring cytogenetic abnormality in association with the blast phase of CML in three patients. This finding will provide a focus for molecular studies aimed at understanding the conversion of CML from chronic to blast phase.

MATERIALS AND METHODS

The patients we studied had been referred to the University of Chicago or Michael Reese Hospitals; specimens of bone marrow and/or blood were analyzed in the Hematology/Oncology Cytogenetics Laboratory. Each patient presented with clinical and morphological features typical of CML in the chronic phase. The diagnosis of CML was confirmed in all cases by identification of the Philadelphia chromosome in hematopoietic tissue. Morphological characterization of the blast phase was based on examination of peripheral blood smears and of bone marrow biopsy specimens and aspirates obtained before institution of acute phase therapy.

Cytogenetic analyses using trypsin-Giemsa, quinacrine fluorescence, and chromomycin-A_3-methyl green reverse fluorescence banding techniques were performed on bone marrow aspirates or peripheral blood samples. All three patients were studied during the blast phase; patient 1 was also studied during the chronic and accelerated phases. Metaphase cells from short-term (24-hour) unstimulated cultures and methotrexate-synchronized cells cultured for 48 hours in phytohemagglutinin (PHA)-stimulated leukocyte-conditioned medium were analyzed. The criteria first proposed by Rowley and Potter[20] and accepted at the First International Workshop on Chromosomes in Leukemia[21] were used for identification of abnormal clones. Chromosomal abnormalities were described according to the ISCN (1985).[22]

Southern blot analysis of DNA from patient 1 was performed as previously described[23]; we used DNA probes specific for the human transferrin receptor (*TFRC*) and Hu-*ets*-2 (*ETS2*) genes. The pTR48 probe contains ~1.6 kilobases (kb) of the 3′ untranslated region of a human *TFRC* cDNA clone[24]; the pTR1/7 probe contains 2.5 kb from the *TFRC* cDNA, including the entire coding region; and the pH33 probe contains 1.0 kb of human genomic sequences from the *ETS2* locus.[25]

RESULTS

Clinical and hematologic features. The clinical and hematologic features of the three patients who had CML and a t(3;21) are summarized in Table 1. All of the patients were men. Each had a relatively long chronic phase during which hydroxyurea and/or busulfan was administered. All three patients had splenomegaly, and patient 2 had lymphadenop-

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Table 1. Summary of Clinical Courses and Hematologic Features of Three Patients With t(3;21)(q26;q22) and CML

Course/Feature	Patient 1	Patient 2	Patient 3
Age/sex	41/M	66/M	30/M
Chronic phase: treatment	Hydroxyurea, Busulfan	Hydroxyurea	Busulfan
Duration (mo)	31	36	60
Accelerated phase			
Manifestations	Weight loss, splenomegaly	—	Fever, pancytopenia
Treatment	Splenectomy	—	Fluoxymesterone
Duration (mo)	14	—	2
Blast phase			
Hemoglobin (g/dl)	12.5	9.0	8.7
Platelets ($\times 10^9$/L)	405	50	13
WBC ($\times 10^9$/L)	86	23	2.3
Blasts (%)	41	61	34
Bone marrow			
Blasts (%)	18	90	80
Morphology	Nonlymphoid	Nonlymphoid*	Nonlymphoid*
TdT	Negative	Negative	Negative
Megakaryocytes			
Number	Increased	Decreased	Decreased
Morphology	Abnormal	Normal*	Normal*
Treatment and response	VP → NR	VPD → NR, DAT → NR	VP → NR, DA,HDAC → NR
Survival (mo)	1	1	2
Overall survival (mo)	46	37	64

Abbreviations: V, vincristine; P, prednisone; D, doxorubicin; A, cytosine arabinoside; T, 6-thioguanine; HDAC, high-dose cytosine arabinoside; NR, no response.

*Bone marrow aspirates could not be obtained because of fibrosis; blast cell features were determined from peripheral blood samples, and megakaryocyte morphology could not be assessed fully.

athy. Patients 1 and 3 had an identifiable accelerated phase, during which patient 1 underwent splenectomy (spleen weight, 3.16 kg at removal) and patient 3 was treated with fluoxymesterone. Following onset of the blast phase, each patient died within 2 months despite vigorous attempts at remission induction.

Blast cells from the bone marrow (patient 1) or peripheral blood (patients 2 and 3) at the time of blast phase had a nonlymphoid morphology and were terminal deoxynucleotidyl transferase (TdT)-negative in each case. Patient 1 had a relatively high platelet count of 405×10^9/L during the blast phase, and his bone marrow and blood contained large numbers of micromegakaryocytes. The platelet count was not elevated in either of the other two patients.

Cytogenetic analyses. The results of cytogenetic analyses are summarized in Table 2 and are illustrated in Fig 1. Patient 1 was studied at three times during the course of his disease. The first study was performed during the chronic phase, 19 months following diagnosis. All 19 bone marrow cells examined had only a typical t(9;22)(q34;q11). The patient subsequently entered an accelerated phase characterized by weight loss and increasing splenomegaly, which was

Table 2. Results of Cytogenetic Studies of Three Patients With t(3;21)(q26;q22) and CML

Patient No.	Phase of Disease	Source	No. of Metaphase Cells Examined	Karyotype
1	Chronic	BM, 24 h	9	46,XY,t(9;22)(q34;q11)(100%)
		BM, 48 h MTX	10	
	Accelerated	BM, 48 h MTX	13	46,XY,t(9;22)(q34;q11)(100%)
	Blast	PB, 24 h	13	46,XY,t(9;22)(q34;q11)(46%)/46,XY,t(3;21)(q26;q22),t(9;22)(q34;q11)(54%)
2	Blast	PB, 24 h	22	48,Y,t(X;9;22)(p22;q34;q11),+8,+11,i(8q),t(3;21)(q26;q22)(95%)/49,Y,t(X;9;22)(p22;q34;q11),+8,+11,i(8q),t(3;21)(q26;q22),+der(22)t(9;22)(q34;q11)(5%)
3	Blast	PB, 24 h	19	46,XY,t(3;21)(q26;q22),t(9;22)(q34;q11)(16%)/48,XY,+15,t(3;21)(q26;q22),t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11)(68%)/48,XY,+15,del(7)(q32q36),t(3;21)(q26;q22),t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11)(16%)

Abbreviations: PB, peripheral blood; BM, bone marrow aspirate; 24 h, cells cultured for 24 hours; 48 h MTX, cells cultured for 48 hours in phytohemagglutinin-stimulated leukocyte-conditioned medium and synchronized with methotrexate.

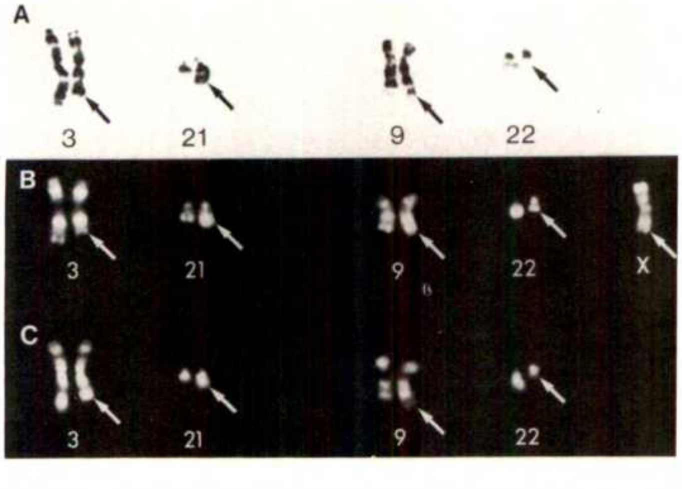

Fig 1. Partial karyotypes of three metaphase cells demonstrating the t(3;21) and the t(9;22) or t(X;9;22). The rearranged chromosomes are located on the right of each pair of homologs and are identified with arrows. (A) Patient 1: G-bands by trypsin using Giemsa. (B) Patient 2: R-bands by fluorescence using chromomycin-A$_3$-methyl green. (C) Patient 3: Q-bands by fluorescence using quinacrine.

refractory to systemic oral chemotherapy. Still, all 13 bone marrow metaphase cells studied had only a t(9;22). At the time of blast phase, however, two related pseudodiploid lines were present; 6 of 13 metaphase cells from the peripheral blood contained a t(9;22) as the only abnormality, and the remaining 7 contained a t(3;21)(q26;q22) in addition to the t(9;22).

Peripheral blood from patients 2 and 3 was studied in our laboratory at the time of blast phase. In patient 2, the predominant clone, comprising 21 of 22 metaphase cells studied, had 48 chromosomes. A three-way translocation, t(X;9;22)(p22;q34;q11), which is a variant of the standard t(9;22), was present. In addition, these cells had extra chromosomes 8 and 11, an isochromosome of the long arm of one chromosome 8 homolog, and a t(3;21)(q26;q22). The remaining cell had 49 chromosomes and contained these abnormalities as well as a second Ph^1. Three related subclones were found in patient 3; one was pseudodiploid and the other two were hyperdiploid. The simplest of these subclones had 46 chromosomes and had a standard t(9;22) and a t(3;21)(q26;q22) as the only abnormalities. The two other subclones had undergone further evolution, with one containing an extra chromosome 15 and a second Ph^1, and the other containing these abnormalities plus a deletion of a part of the long arm of one chromosome No. 7 [del(7)(q32q36)].

DNA analysis. At the cytogenetic level of resolution, the locations of the breakpoints of the t(3;21) at bands 3q26 and 21q22 correspond to the mapped positions of the *TFRC* gene[26] and of the human homolog 2 of the *ets* sequence of the transforming avian erythroblastosis virus E26 (*ETS2*),[27] respectively. To determine whether the *TFRC* or *ETS2* gene loci were rearranged as a consequence of the translocation, we performed Southern blot analysis of DNA from peripheral blood cells of patient 1; placental DNA was used as a control. DNA, which had been digested with *Eco*RI, *Hind*III, or *Pvu*II, was hybridized sequentially to two *TFRC* probes and an *ETS2* probe. The sample had been obtained at blast phase, concurrently with the sample used for cytogenetic analysis. The WBC count at that time was 86.4 × 10^9/L; of these cells, 41% were blasts and 36% were micromegakaryocytes. Only germline restriction fragments were detected with the probes. Thus, in this patient, there was no evidence for a translocation breakpoint within the *TFRC* or *ETS2* genes.

DISCUSSION

We identified an identical t(3;21)(q26;q22) in three male patients with Philadelphia chromosome-positive CML during blast phase. In one patient, whom we studied serially, we showed that the translocation has occurred in association with the development of the blast phase as the result of clonal karyotypic evolution. We have not observed a t(3;21) in >500 other patients with CML in the chronic phase (M. M. Le Beau and J. D. Rowley, unpublished observations). A review of the literature and of Mitelman's *Catalogue of Chromosome Aberrations in Cancer*[28] failed to reveal any other cases with a t(3;21)(q26;q22). One patient has been reported as having a t(3;21)(q12;q22) in the blast phase of CML[29]; however, the breakpoint on chromosome 3 differs from that in our cases. Thus, we have identified a new recurring abnormality associated with the blast phase of CML.

The t(3;21) is not entirely specific for CML, however; the same rearrangement has been observed as the only abnormality in bone marrow from two other patients studied in our laboratory, both of whom developed a myelodysplastic syndrome following cytotoxic chemotherapy for a prior malignancy (M. M. Le Beau and J. D. Rowley, unpublished observations). Because the t(3;21) is likely to be therapy induced in these two patients, the same abnormality found in the patients with CML may also be a result of exposure to mutagenic agents rather than a spontaneous evolutionary event. All three patients with CML and a t(3;21) had received chemotherapy during the chronic phase; two of these received the alkylating agent busulfan.

The breakpoints of the t(3;21) are of interest with respect to previously identified cytogenetic abnormalities in leukemia. The breakpoint in 3q26 corresponds to one of the breakpoints in the t(3;3)(q21;q26) and the inv(3)(q21q26), both of which are associated with ANLL and abnormal megakaryocytopoiesis.[15,16,30,31] Patient 1 had a high platelet count and numerous micromegakaryocytes in his bone marrow in the blast phase, reminiscent of the findings in ANLL patients with t(3;3) or inv(3). In contrast, patients 2 and 3 had low platelet counts and a marked reduction of morphologically identifiable megakaryocytes in the marrow at blast phase, suggesting that factors other than a breakpoint at 3q26 are important in determining the presence of megakaryocyte abnormalities. A breakpoint in 3q21, rather than 3q26, in the blast phase of CML has been proposed to be associated with a high platelet count and abnormal megakaryocytes. Bernstein et al reported two patients who had secondary cytogenetic abnormalities involving 3q21 at the time of blast phase of CML.[17] One had an inv(3)(q21q26) like that seen in ANLL, and the other had a t(3;9)(q21;q34); both had elevated platelet counts and numerous micromegakaryocytes at the time of blast phase. The common breakpoint in these two cases is 3q21. In the present report, the findings in patient 1 suggest that a breakpoint at band 3q26 without a 3q21 abnormality may also influence the megakaryocytes in certain cases; however, additional examples are needed for confirmation of this possibility.

The breakpoint in the t(3;21) at band 21q22 also corresponds to one breakpoint in another specific abnormality found in ANLL, namely, the t(8;21)(q22;q22).[32] The t(8;21) has not been identified in any reported cases of CML. Alimena et al, however, tabulated 256 breakpoints observed during blast phase of CML and found a cluster of eight occurring at 21q22,[33] suggesting that this may be an important site with respect to progression of CML.

By analogy to other recurring chromosomal rearrangements in human leukemia that have been studied at the molecular level, the consistency of the breakpoints in the t(3;21) implies that specific genes in bands 3q26 and 21q22 participate in this rearrangement. Furthermore, the t(3;21) may play an important role in disease progression. The latter is suggested by the absence of the t(3;21) during the chronic phase in patient 1 and by the presence of the t(3;21) at blast phase as the only chromosomal rearrangement other than the t(9;22) in the primary clone in patients 1 and 3. Therefore, an investigation of this abnormality at the DNA level might be helpful for an understanding of the basis for the conversion of CML from chronic to blast phase. Our initial approach has been to determine by standard Southern blot analysis whether *TFRC* or *ETS2*, which have been localized near bands 3q26[26] and 21q22,[27] respectively, was rearranged in DNA from patient 1. By using this method, we did not find abnormalities of either gene; however, new techniques such as pulsed-field gel electrophoresis, by which one can examine very large regions of DNA, may allow detection of rearrangements located farther away from these genes.[34]

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Recombinant Gibbon Interleukin 3 Supports Formation of Human Multilineage Colonies and Blast Cell Colonies in Culture: Comparison With Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor

By Anne G. Leary, Yu-Chung Yang, Steven C. Clark, Judith C. Gasson, David W. Golde, and Makio Ogawa

The genetic sequences encoding the gibbon and human interleukin 3 (IL 3) proteins were molecularly cloned. The amino acid sequence of the mature gibbon IL 3 protein proved to share 93% homology with the corresponding human protein. We examined the effects of biosynthetic (recombinant) gibbon IL 3 on the proliferation and differentiation of an enriched population of human hematopoietic progenitors and compared the results with the effects of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). Gibbon IL 3 supported the formation of various types of single lineage as well as multilineage colonies by My-10$^+$ bone marrow cells in the presence of human erythropoietin (Ep). In contrast, recombinant human GM-CSF supported the formation of single-lineage colonies and only a small number of multilineage colonies. Both IL 3 and GM-CSF had significant erythroid burst-promoting activity (BPA). Delayed addition of gibbon IL 3 to low serum culture of My-10$^+$ marrow cells supported the formation of blast cell colonies with variable but high replating capability. Human GM-CSF was less effective than IL 3 in support of multipotential blast cell colonies. These results are analogous to the effects of murine IL 3 and GM-CSF on murine progenitors and support the notion that the primary factor for multipotential progenitors is IL 3.

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HEMATOPOIETIC CELL proliferation and differentiation in semisolid culture is supported by protein/glycoprotein hormones called colony-stimulating factors (CSFs). CSFs are produced by a variety of tissues and include CSF-1, a macrophage CSF,[1] granulocyte-macrophage CSF (GM-CSF),[2] granulocyte CSF (G-CSF)[3] and interleukin 3 (IL 3).[4] These CSFs have been purified, and the genes encoding the proteins have been molecularly cloned. Whereas CSF-1 and G-CSF act primarily on monopotent progenitors, murine IL 3 and GM-CSF have been reported to support proliferation of multipotential progenitors. Murine IL 3 was purified by Ihle et al[4] in 1982 and subsequently was shown to support formation of multilineage colonies[5] and blast cell colonies in culture.[6,7] Recently, the genes for gibbon and human IL 3 were identified using expression cloning techniques, and the gibbon IL 3 has been expressed in Cos-1 monkey cells.[8] Comparison of the amino acid sequences of the human and gibbon proteins revealed that they are 93% homologous.[8] This extensive homology suggests that gibbon and human IL 3 may be functionally similar.

Using granulocyte-macrophage colony formation in agar as an assay, murine GM-CSF was initially purified from medium conditioned by mouse lung tissue.[2] Subsequent studies carried out by Metcalf et al[9] and in our laboratory[10] suggested that it is capable of supporting proliferation of multipotential progenitors. Emerson et al[11] reported that human GM-CSF supports proliferation of multipotential progenitors. In this report, we examined the effects of gibbon recombinant IL 3 on hematopoietic colony formation by highly enriched human bone marrow cells and compared the results with the effects of human recombinant GM-CSF in culture.

MATERIALS AND METHODS

Recombinant factors. The recombinant gibbon IL 3 used in this study was obtained by transfecting monkey Cos-1 cells with gibbon IL 3 cDNA clone, pCSF-MLA.[8] Five micrograms of pCSF-MLA DNA was used to transfect a 100-mm dish of Cos-1 cells by diethylaminoethanol (DEAE) Dextran–mediated DNA transfection with the addition of 0.1 mmol/L chloroquine.[12,13] Conditioned media (CM) from transfected Cos-1 cells were harvested 72 hours after transfection. The concentration of IL 3 was estimated to be 100 nmol/L according to the bioassay developed by Griffin et al.[14]

Human GM-CSF was purified as previously described[15,16] from serum-free medium conditioned by monkey Cos-1 cells transfected with human GM-CSF cDNA clone, p91023(B). The purified GM-CSF appeared as a single protein band of 22,000 mol wt on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the same mol wt as the purified natural GM-CSF.[15] Protein concentration was estimated by amino acid analysis.

Methylcellulose cell culture. Bone marrow cells were aspirated from the posterior iliac crest of healthy adult volunteers and placed in 16-mL Falcon tissue culture tubes containing 400 U of preservative-free heparin (Invenex Laboratories, Melrose Park, IL). The donors were advised of the risks of marrow aspiration and gave informed consent. These studies were approved by the Institutional Review Board for Human Research. Mononuclear cells were isolated by use of Ficoll-Paque (Pharmacia, Piscataway, NJ), and the nonadherent fraction was separated by overnight adherence to plastic. Samples were further enriched for colony-forming cells by positive indirect immune adherence using human progenitor cell antibody (HPCA) (Becton Dickinson, Mountain View, CA) which is the commercial preparation of monoclonal anti–My-10 antibody,[17] and the "panning" technique of Engleman et al.[18] Details of this procedure have been reported previously.[17]

Panned My-10$^+$ bone marrow cells were cultured in 35-mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique of Iscove et al.[19] One milliliter of

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culture consisted of 2,000 My-10$^+$ bone marrow cells, α-medium (Flow Laboratories, Rockville, MD), 1.2% of 1,500 centipoise methylcellulose (Fisher Scientific, Norcross, GA), 30% fetal calf serum (FCS) (Flow Laboratories), 1% deionized bovine serum albumin (BSA) (Sigma Chemical, St Louis), 5×10^{-5} mol/L mercaptoethanol, 2 U/mL human urinary erythropoietin (Ep) with a specific activity of 370 U/mg protein (kindly provided by Dr Makoto Kawakita, Kumamoto University School of Medicine, Japan) and variable concentrations of IL 3 and GM-CSF. Dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. On days 14 through 16 of incubation, the types and sizes of the colonies were estimated in situ. Abbreviations for colony types were: GM, granulocyte and/or macrophage colonies; Meg, megakaryocyte colonies; Eo/Baso, eosinophil and/or basophil colonies;[20] E, erythroid bursts; and E-mix, mixed colonies containing erythroid cells. When cytologic confirmation was required, colonies were individually lifted from methylcellulose by an Eppendorf micropipette and slides were prepared using a cytospin centrifuge (Shandon Southern Instruments, Sewickley, PA). Slides were stained with May-Grunwald-Giemsa (Gallard Schlesinger Chemical, Carle Place, NY) for differential counting.[21]

Blast cell colony formation. The factors were also tested for their capacity to support blast cell colony formation using a technique we developed recently.[22] In brief, 1×10^4 panned My-10$^+$ bone marrow cells were plated in methylcellulose culture containing: α-medium; 2% FCS; 1% crystallized, deionized BSA; 5×10^{-5} mol/L mercaptoethanol; and 300 μg/mL fully iron saturated human transferrin (98% pure) 160 μg/mL soybean lecithin 96 μg/mL cholesterol, and 10^{-7} mol/L sodium selenite (Sigma). On day 14 of incubation, 0.1 mL of a 1:50 dilution of IL 3 or 10 or 1 nmol/L GM-CSF was layered over the culture, and the dishes were examined daily for the appearance of blast cell colonies between days 21 and 32.

When identified, blast cell colonies were replated in culture for confirmation of their ability to form secondary colonies. For these experiments, blast cell colonies were individually lifted from the culture and suspended in 100 μL α-medium. The samples were gently pipetted and then added to 0.9 mL methylcellulose medium containing 30% FCS, 1% deionized BSA, 2 U/mL Ep, and 30% C5MJ CM.[22] Cultures were incubated at 37°C and 5% CO_2 in air. When colonies appeared to have matured (generally after 12 to 16 days of culture), individual colonies were prepared for differential counting as descibed above.

RESULTS

Colony formation supported by IL 3 and/or GM-CSF. First, we carried out titration of IL 3 for colony formation in culture. Two thousand My-10$^+$ marrow cells were plated in cultures containing different dilutions of IL 3 preparations and 2 U/mL Ep. As controls, cultures were also prepared with conditioned medium from nontransfected Cos-1 cells (mock) plus Ep, C5MJ CM plus Ep, or Ep alone. C5MJ supports a wide variety of colony types, including mixed colonies.[22] The results are presented in Table 1. IL 3 preparations ranging from 1:50 dilutions to 1:5,000 dilutions supported formation of various types of single lineage as well as multilineage colonies. Although the number of colonies supported by three different dilutions of IL 3 were similar, the colonies were smaller in the 1:5,000 dilution of IL 3 than those grown in higher concentrations of IL 3. Most notably, the largest colonies in all categories were smaller in 1:5,000 dilutions than in 1:500 dilutions. Based on these observations, the 1:500 dilution was chosen as the standard concentration. The concentration of IL 3 in this dilution was estimated to be 200 pmol/L (described in the Materials and Methods section). The mock CM plus Ep or Ep alone supported only occasional GM colonies or clusters in the My-10$^+$ fraction of cells.

In the next experiment, My-10$^+$ marrow cells were plated in quadruplicate cultures in the presence of Ep, GM-CSF, and/or IL 3. The results are presented in Table 2. To confirm that 100 pmol/L GM-CSF is the optimal concentration for colony formation, marrow cells were cultured in five different concentrations of GM-CSF. As has been shown by Tomonaga et al,[23] this concentration of GM-CSF was the optimal concentration for colony formation. A 1:500 dilution (~200 pmol/L) of IL 3 and Ep supported formation of various types of colonies, including E-mix colonies. A representative mixed colony containing macrophages, eosinophils, and erythrocytes is shown in Fig 1. In contrast, GM-CSF rarely supported formation of E-mix colonies. As demonstrated by a number of investigators,[24-26] GM-CSF in all concentrations augmented formation of erythroid bursts in culture. Mock CM and Ep failed to support formation of erythroid bursts.

Cytologic analysis of lineage expression. For determination of the precise lineages expressed in the colonies in culture, 2,000 My-10$^+$ marrow cells were plated in each dish with IL 3 plus Ep or GM-CSF plus Ep, and on days 14 through 16 of incubation, colonies in each group were individually analyzed for their cellular composition. The results are presented in Table 3. In two separate experiments, most colonies supported by GM-CSF were single-lineage colonies. A 100-fold increase in the concentration of GM-

Table 1. Colony Formation From MY-10$^+$ Marrow Cells Supported by IL 3 and Ep

Factors	GM	EO/BASO	E	E-MIX	Total
C5MJ CM	14	4	13	5	36
	(50-700)*	(1,000-2,000)	(100-4,000)	(500-3,000)	
IL 3 (1:50)	22	5	13	4	44
	(50-700)	(100-1,000)	(200-2,500)	(1,000-2,000)	
IL 3 (1:500)	23	6	11	4	44
	(50-2,000)	(300-2,000)	(300-2,000)	(200-5,000)	
IL 3 (1:5,000)	26	4	13	3	46
	(30-300)	(100-1,000)	(100-1,000)	(300-1,000)	

Average of colony numbers in duplicate cultures. Concentrations of IL 3 ranged from 1:50 to 1:5,000 dilutions, and Ep was constant at 2 U/mL.

*Numbers in brackets represent range of estimated size of colonies.

Table 2. Colony Formation From MY-10$^+$ Marrow Cells Supported by Ep and GM-CSF or IL 3

Factors	GM	E	EO/BASO	E-MIX	MEG	Total
GM-CSF (10 nmol/L)	26 ± 3	6 ± 2	6 ± 3	0	2 ± 1	40 ± 3
GM-CSF (1 nmol/L)	26 ± 3	5 ± 2	6 ± 1	1 ± 1	3 ± 1	40 ± 2
GM-CSF (100 pmol/L)	31 ± 6	9 ± 1	6 ± 2	1 ± 1	2 ± 1	49 ± 5
GM-CSF (10 pmol/L)	20 ± 2	8 ± 3	5 ± 2	1 ± 1	3 ± 2	37 ± 5
GM-CSF (1 pmol/L)	16 ± 3	8 ± 1	2 ± 1	1 ± 1	1 ± 1	28 ± 4
IL 3 (1:500)*	21 ± 2	8 ± 1	5 ± 2	7 ± 1	1 ± 1	42 ± 2
Mock conditioned medium	3 ± 1	0	0	0	0	3 ± 1
C5MJ conditioned medium	42 ± 5	8 ± 1	4 ± 1	7 ± 1	1 ± 1	62 ± 8

Mean ± SD of colony numbers per dish in quadruplicate cultures. Concentration of Ep was 2.0 U/mL.

*Concentration of IL 3 was estimated to be 200 pmol/L.

CSF showed no difference in the number and types of colonies. IL 3 plus EP supported formation of many types of multilineage colonies, including pentalineage colonies. In contrast, only a few mixed colonies were supported by GM-CSF plus Ep.

Blast cell colony formation. In 8 experiments using 8 separate marrow samples, 67 blast cell colonies were identified in 50 culture dishes receiving delayed addition of IL 3. Thirty-one of 48 replated blast cell colonies yielded secondary colonies in the presence of C5MJ CM plus Ep. The size of blast cell colonies ranged from 21 cells to 41 cells/colony, and the percentage of replating efficiencies of the positive colonies ranged from 11% to 65%. As shown in Table 4, secondary colonies included single-lineage colonies such as neutrophil (n), macrophage (m), eosinophil (e), basophil (b), or erythrocyte (E) colonies as well as many bilineage and trilineage colonies. Occasional tetralineage secondary colonies revealed all four leukocyte lineages. Two concentrations of GM-CSF were tested for their ability to support blast cell colony formation. When 13 blast cell colonies were replated in C5MJ CM plus Ep, only two colonies produced secondary colonies revealing n, m, or nm lineages. When a tenfold higher concentration of GM-CSF was used, four of ten blast cell colonies produced secondary colonies, including eosinophil colonies. Most blast cell colonies supported by GM-CSF apparently do not possess significant proliferative capacity and probably consist of progenitors committed only to the n, m, and e pathways.

DISCUSSION

Despite extensive search by a number of investigators, the gene for human IL 3 eluded identification until recently. Using ^{3}H-labeled thymidine uptake by blast cells from patients with chronic myelogenous leukemia[14] as an assay in expression cloning, an IL 3 gene was identified from a cDNA library prepared from a gibbon lymphosarcoma cell line.[8] This gibbon cDNA was used as a probe to identify and clone the gene for human IL 3.[8] The nucleotide sequence of the coding region of gibbon IL 3 proved to share 96% homology with the human IL 3 sequence.[8] From this observation it was deduced that the protein from the two species differ by 11 amino acids, 9 of which reside in the mature protein sequence. We found gibbon IL 3 to be active on purified human progenitors. The results presented in this article suggest that the function of gibbon IL 3 will be similar to human IL 3 since in the presence of Ep gibbon IL 3 supported formation of all types of single-lineage colonies, multilineage colonies, and multipotential blast cell colonies.

A controversy exists regarding the targets of murine and human GM-CSF. Murine GM-CSF was originally consid-

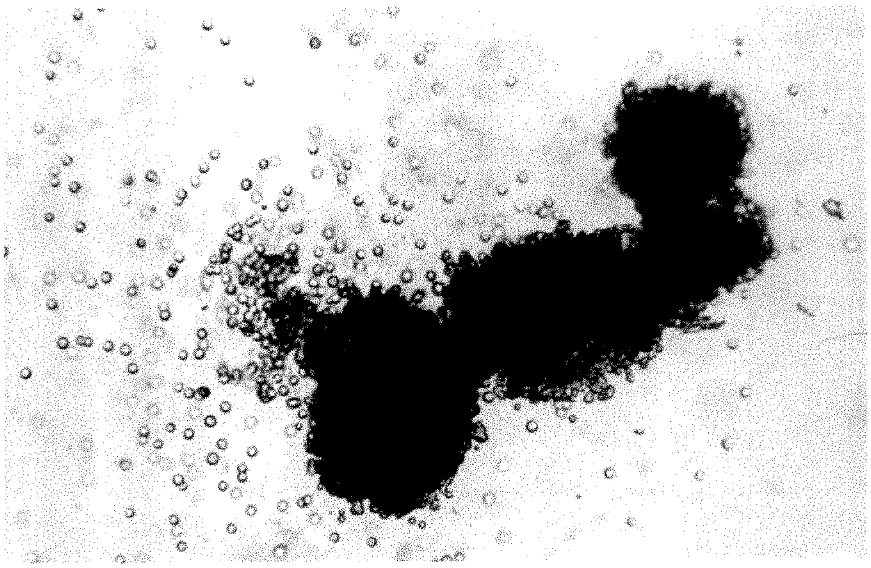

Fig 1. A typical mixed colony supported by IL 3 contains macrophages, eosinophils, and erythrocytes.

Table 3. Cytologic Analysis of Lineages Expressed by Colonies Supported by IL 3 Plus Ep or GM-CSF Plus Ep

Lineages						Sample 1			Sample 2			
							GM-CSF			GM-CSF		
n	m	b	e	E	M	IL 3	1 nmol/L	100 pmol/L	IL 3	10 nmol/L	1 nmol/L	100 pmol/L
+								1	1	1	2	1
	+					18	16	18	14	17	20	18
		+					1			2	1	1
			+			2	2	2	3	5	4	7
				+		4	5	5	5	8	7	9
					+	2	3	2	2	3	4	3
+	+						2		3	2	3	3
	+	+								1		
	+		+			1				1		
	+			+		1			4	1		2
	+				+	2	1					
			+	+		1			1			
				+	+	1		1				
+	+		+						2		1	
+	+			+		2			1			
+			+	+					1			
	+	+		+		1						
	+		+	+					2			
	+			+	+	1						
	+	+		+	+	1						
	+		+	+	+	1			1			
+	+		+	+	+				1			
	+	+	+	+	+				1			
Total						38	30	29	42	41	42	44

Abbreviation of lineages are as recommended by a UCLA Workshop (30).
All colonies in a single dish containing 2,000 My-10$^+$ cells were individually analyzed.

ered specific for the GM pathways. Most recently, Kajigaya et al[27] reported that recombinant murine GM-CSF is restricted to the GM pathways. In contrast, Metcalf et al in 1980[9] reported that purified murine GM-CSF supports a few cell divisions of multipotential progenitors. They also demonstrated that very high concentrations of recombinant murine GM-CSF support formation of eosinophil colonies, macrophage colonies, and E-mix colonies.[28] Recently, we showed that murine GM-CSF supports formation of multipotential blast cell colonies.[22] Comparison with the blast cell colony formation supported by IL 3, however, suggested that the progenitors for the blast cell colonies that respond to GM-CSF represent only a subpopulation of those responding to IL 3.

Human GM-CSF is generally believed to support proliferation and maturation of neutrophil, macrophage, and eosinophil progenitors.[23,29] Controversy exists regarding additional functions of human GM-CSF in hematopoiesis. Investigators at Harvard University[11,24,25] reported that human GM-CSF is capable of supporting multilineage colony formation and possesses significant erythroid burst potentiating activity (BPA). In contrast, Metcalf et al[29] reported only minimal amounts of such activities. We have shown that human GM-CSF and human Ep supports formation of all types of single-lineage colonies, including basophil and megakaryocyte colonies. Our data corroborated those on human GM-CSF of Kaushansky et al[26] regarding the support for megakaryocyte colony formation. Our observation also corroborates the observation by Donahue et al[24] and Sieff et al[25] that human GM-CSF possesses significant BPA in vitro. The observed phenomena may not be solely due to the direct action of human GM-CSF because the culture media contained FCS, Ep, and BSA. Although My-10$^+$ bone marrow cells were highly enriched for progenitors, they were not a pure population of progenitor cells.

Both IL 3 and GM-CSF supported formation of multilineage colonies. Cytologic examination of individual colonies revealed, however, that gibbon IL 3 is far more effective

Table 4. Summary of Blast Cell Colony Formation by IL 3 or GM-CSF

Factor	Incidence of Blast Cell Colonies	Positive Colonies in C5MJ	Plating Efficiency	Lineages in Secondary Colonies
IL 3*	67 colonies/51 dishes	31/48	11%-65%	n, m, e, b, E, nm, me eb, eE, mE, meE, nmeb
GM-CSF (100 pmol/L)	19 colonies/40 dishes	2/13	14%-20%	n, m, nm
GM-CSF (1 nmol/L)	10 colonies/20 dishes	4/10	18%-63%	n, m, e, nm

*Concentration of IL 3 in each dish was a 1:500 dilution, which is ~200 pmol/L.

than human GM-CSF in supporting the proliferation of multipotential progenitors. Whereas a number of multipotential blast cell colonies were identified in cultures receiving delayed addition of IL 3, fewer blast cell colonies were supported by GM-CSF and yielded secondary colonies. The secondary colonies were restricted to neutrophil, macrophage, and eosinophil lineages. These observations are analogous to the reported results of murine factors on multipotential progenitors.[9,10] Our previous data suggested that the progenitors for murine blast cell colonies responding to murine GM-CSF represent only a subpopulation of those responding to murine IL 3.[10] Human multipotential progenitors that respond to GM-CSF may also represent a small subpopulation of multipotential progenitors sensitive to IL 3.

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Molecular Characterization of Chromosome 7 Long Arm Deletions in Myeloid Disorders

By Juha Kere, Tapani Ruutu, Reino Lahtinen, and Albert de la Chapelle

Partial deletion of the long arm of chromosome 7 is a common abnormality in the bone marrow cells of patients with myelodysplastic syndrome (MDS) or acute nonlymphocytic leukemia (ANLL). This study was undertaken to characterize the chromosome breakpoints in molecular terms and to determine if hemizygosity or submicroscopic deletions occur in patients without any cytogenetically detectable abnormality of chromosome 7. We studied restriction fragment length polymorphisms with 10 chromosome 7-specific DNA probes in separated WBC fractions. No molecular abnormalities occurred in lymphocyte-derived DNA. Several probes located in band 7q22 or distally thereof detected deletion of one allele in granulocyte-derived DNA from all four patients with chromosome 7 long arm deletion. In the granulocytes of one patient heterozygosity for the T cell receptor β chain gene (in band 7q35) indicated that the deletion was interstitial. NJ-3, a proalpha2(I)collagen gene probe (in band 7q21-22) detected heterozygosity in the granulocytes of one patient. No hemizygosity or deletions were found in four patients with two normal chromosomes 7. These results confirm that mature granulocytes but not lymphocytes are derived from the abnormal clone. Interstitial deletions exist, and the extent of deleted genomic material varies among patients.

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PARTIAL DELETION of the long arm of one chromosome 7 is a common recurrent chromosome abnormality in secondary myelodysplastic syndrome (MDS) or acute nonlymphocytic leukemia (ANLL). The abnormality is often designated as del(7)(q22) implicating terminal deletion with breakage in band q22, but other breakpoints and interstitial deletions have been described in many patients.[1,2] In molecular terms, however, the deletions have not yet been characterized. Development of hemizygosity has been demonstrated in solid tumors as a mechanism possibly contributing to the tumorigenesis.[3-8] In this article, we address two questions: First, can the cytogenetically observed deletions be further characterized with molecular methods, and second, can hemizygosity be observed in patients with two cytogenetically normal chromosomes 7. We previously demonstrated that in MDS or ANLL monosomy 7 or partial deletion of the long arm can be detected in blood granulocytes or monocytes with restriction fragment length polymorphism (RFLP) analysis.[9] In the present study, we tested 10 DNA probes located in different parts of chromosome 7 for their presence or absence in each of the two chromosomes 7. We studied four patients with a 7q− chromosome to characterize the chromosomal breakpoints in molecular terms, and four patients with two apparently normal chromosomes 7 to determine if development of hemizygosity or deletions can be detected. Our results provide basic knowledge about the chromosomal breakpoints in molecular terms and confirm heterogeneity of the 7q− chromosome in myeloid disorders.

MATERIALS AND METHODS

Patients and cytogenetic studies. Eight patients with a hematologic disorder were studied (Table 1). The study was approved by the ethics committee of the Department of Medical Genetics, University of Helsinki. Cytogenetic studies of blood and bone marrow specimens were performed using standard techniques and Giemsa banding as previously described.[13]

Specimens and cell separation. Blood (10 to 40 mL) was diluted with phosphate-buffered saline (PBS) solution, and the mononuclear cells were separated from granulocytes and RBCs within 24 hours of sampling by Ficoll density-gradient centrifugation.[14] The lymphocyte and granulocyte fractions were recovered as described.[9] Purity of the cell fractions was checked from smears or cytocentrifuge preparations stained with May-Grünwald-Giemsa or Sudan black B.

DNA probes and molecular studies. High-mol-wt DNA was extracted separately from each of the blood cell fractions. A standard proteinase K digestion, phenol extraction, and ethanol precipitation procedure was used.[15] The RFLP analysis was done using previously published methods with minor modifications.[16] For each experiment, 3 to 5 μg DNA was digested with a restriction enzyme according to the instructions. Whole blood DNA samples from healthy subjects were included in each experiment as controls. Completeness of the digests was confirmed by staining the gels with ethidium bromide. Nitrocellulose filters (Schleicher & Schuell, Dassel, FRG) were used in Southern blotting. Nick-translation of the DNA probes was done using an Amersham N.5000 kit. Hybridization was at 65°C for 19 to 24 hours. The autoradiograms were scanned using a laser densitometer (LKB 2202 Ultroscan, Bromma, Sweden) to quantitate the strength of the allelic bands.

The chromosome 7-specific DNA probes used in this study are listed in Table 2. All patients were tested for RFLPs using whole blood-, lymphocyte-, and granulocyte-derived DNA samples.

RESULTS

Cytogenetic studies. By conventional cytogenetic analysis of bone marrow mitoses, patients 1 through 4 each had a partial deletion of chromosome 7 in addition to other abnormalities (Table 1, Fig 1). In patients 1 and 2, only the abnormal clone was found; in patients 3 and 4, mitoses with a normal karyotype also occurred. Patients 5 through 8 had

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Table 1. Clinical and Cytogenetic Findings in Patients

Patient	Sex/Age at Study	Diagnosis[10,11] Age at Diagnosis	History of Exposure	Bone Marrow Karyotype[12] at Time of Study
1	F/77	RAEB/72	CT, RP*	46, XX, 7q−, del(20)(q11) = 16
2	M/82	RAEB/80	none	46, XY, t(2;13)(p21;q12), 7q− = 20
3	M/38	ANLL M2/38	CT, RT*	46, XY = 5/46, XY, 7q− = 15/46, XY, 7q−, del(16)(q22) = 2
4	F/40	RAEB/40	CT, RT*	46, XX = 2/44, XX, −17, −21, del(5)(q13q22), 7q−, del(13)(q12q21), del(20)(q11) = 21
5	M/61	RA/60	none	46, XY = 11/46, XY, del(13)(q14q21) = 3
6	M/61	ANLL M2/61	none	46, XY = 1/47, XY, +8 = 8
7	F/51	RA/51	none	47, XX, +8 = 20
8	F/77	PV/77	none	48, XX, +8, +9 = 22

CT, chemotherapy; RT, radiotherapy; RP, radiophosphorus; PV, polycythemia vera.

*Patient 1 had PV; patients 3 and 4 were previously treated for lymphoma.

other chromosomal abnormalities in the bone marrow but two apparently normal chromosomes 7. In patients 3 and 4, the deletion was cytogenetically compatible with the designation del(7)(q22); this implies a terminal deletion, but the presence or absence of a telomeric piece cannot be definitely ascertained. In patient 2, the deleted chromosome looks longer and could be interstitial with breakpoints at 7q22 and q33-34. In patient 1, the deletion also appeared different and an interstitial deletion del(7)(pter → q21::q32-35 → qter) was suspected. To accommodate the uncertainty as to the cytogenetic definition of the breakpoints, all partially deleted chromosomes 7 were designated 7q−. Phytohemagglutinin (PHA)-stimulated blood mitoses were normal in all patients.

Purity of separated cell fractions. The mononuclear nonadherent cell ("lymphocyte") fraction contained 80% to 96% lymphocytes in different experiments. In the granulocyte fraction 66% to 99% of the nucleated cells were neutrophils in different experiments. The fractions were cross-contaminated; <9% erythroblasts were present in the lymphocyte fractions. The purity of each sample is given in the legend to Fig 2.

RFLP heterozygosity. All patients were heterozygous for at least one polymorphism. The constitutional RFLP alleles are shown in Table 3. No differences were observed between the whole blood-, lymphocyte-, and granulocyte-derived DNA samples from patients 5-8.

Table 2. Chromosome 7-Specific DNA Probes, Proposed Localization of Sequences in Chromosome 7, and Restriction Fragment Length Polymorphisms

Probe	Gene or Locus	Localization	Restriction Fragment Length Polymorphism		Reference
			Enzyme	Allele Size (Kbp)	
ASSpC7	*ASSP11*	7p15	*Hind*II	10.1, 5.3	17
NJ-1	*COL1A2*	7q21-22	*Msp*I	2.1, 1.6	18-20
NJ-3	*COL1A2*	7q21-22	*Eco*RI	13.0, 9.5	18-20
B79a	*D7S13*	7q22	*Msp*I	11.6, 8.4	21
MetH	*MET*	7q22	*Msp*I	4.8, 2.3, 1.8	22
MetH	*MET*	7q22	*Taq*I	7.5, 4.0	22
MetD	*MET*	7q22	*Taq*I	6.0, 4.4	22
J3.11	*D7S8*	7q22	*Msp*I	4.1, 1.7	23
7C22	*D7S16*	7q31	*Eco*RI	7.2, 5.1	24
hR-II		7q35	*Ban*II	7.6, 4.8	25
TCRB	*TCRB*	7q35	*Bgl*II	11.5, 10.0	26-28

Missing RFLP alleles in patients with deletions. One of the *MET* oncogene alleles was very weak or almost absent in the granulocyte fractions of patients 1 through 3 as detected by the MetH probe. In patient 4, the 1.7-kilobase (kb) allele detected by probe J3.11 was very weak in the granulocyte fraction. Marked reduction in the intensity of one of the allelic bands in the granulocyte fraction was also observed with probe B79a in patients 1 and 2, with probe 7C22 in patients 2 and 3, with probe hR-II in patient 2, and with probe TCRB in patient 1. The visual impression was confirmed by densitometry in all cases. The allelic bands of patient-derived DNA samples were compared with those of healthy controls, and the reduction in intensity was 69% to 98% (35% to 62% with probe 7C22) in different samples. In the whole blood-derived DNA, the weaker allele was always stronger than in the granulocyte DNA but weaker than in the lymphocyte DNA. In contrast, two alleles of normal strength were observed in the granulocyte-derived DNA samples of patients 1 and 3 with probe ASSpC7 (located in the short arm), of patient 2 with probe NJ-3, and of patient 1 with probe TCRB. The results are shown in Fig 2. No unexpected hybridization bands suggesting rearrangements were observed.

DISCUSSION

Loss of an RFLP allele in the malignant tissue has been previously observed in several tumors and hematologic malignancies.[3-9,29] The loss may be due to monosomy or deletion of part of a chromosome or to hemizygosity without visible cytogenetic changes on the molecular level. Weakness or absence of chromosome 7-specific RFLP alleles was observed in all four patients with a 7q− chromosome. In contrast, none of the four patients with two apparently normal chromosomes 7 showed weakness of one allele. These results are compatible with the cytogenetic analyses of bone marrow and PHA-stimulated whole blood cultures. We conclude that loss of alleles in the granulocyte fractions was due to the presence of a 7q− chromosome in most if not all of the blood granulocytes. Weakness instead of absence of allelic bands in the granulocyte-derived samples was probably due to contamination of the granulocytes with other blood cells with two chromosomes 7 as shown by the cytologic stainings (described in legend to Fig 2). Another explanation is the coexistence of subsets of granulocytes with

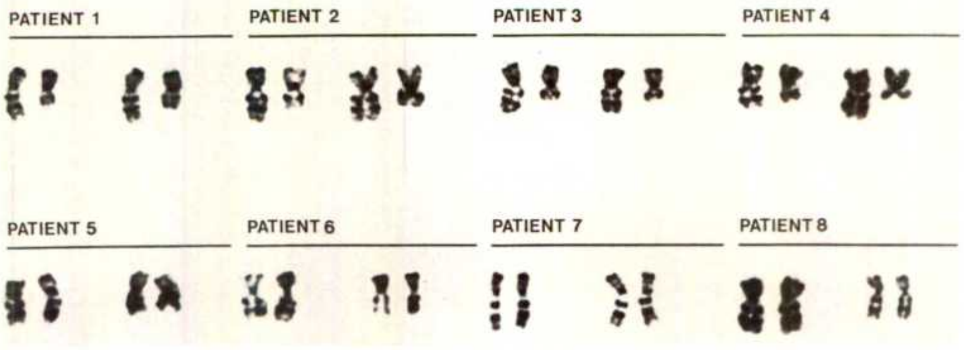
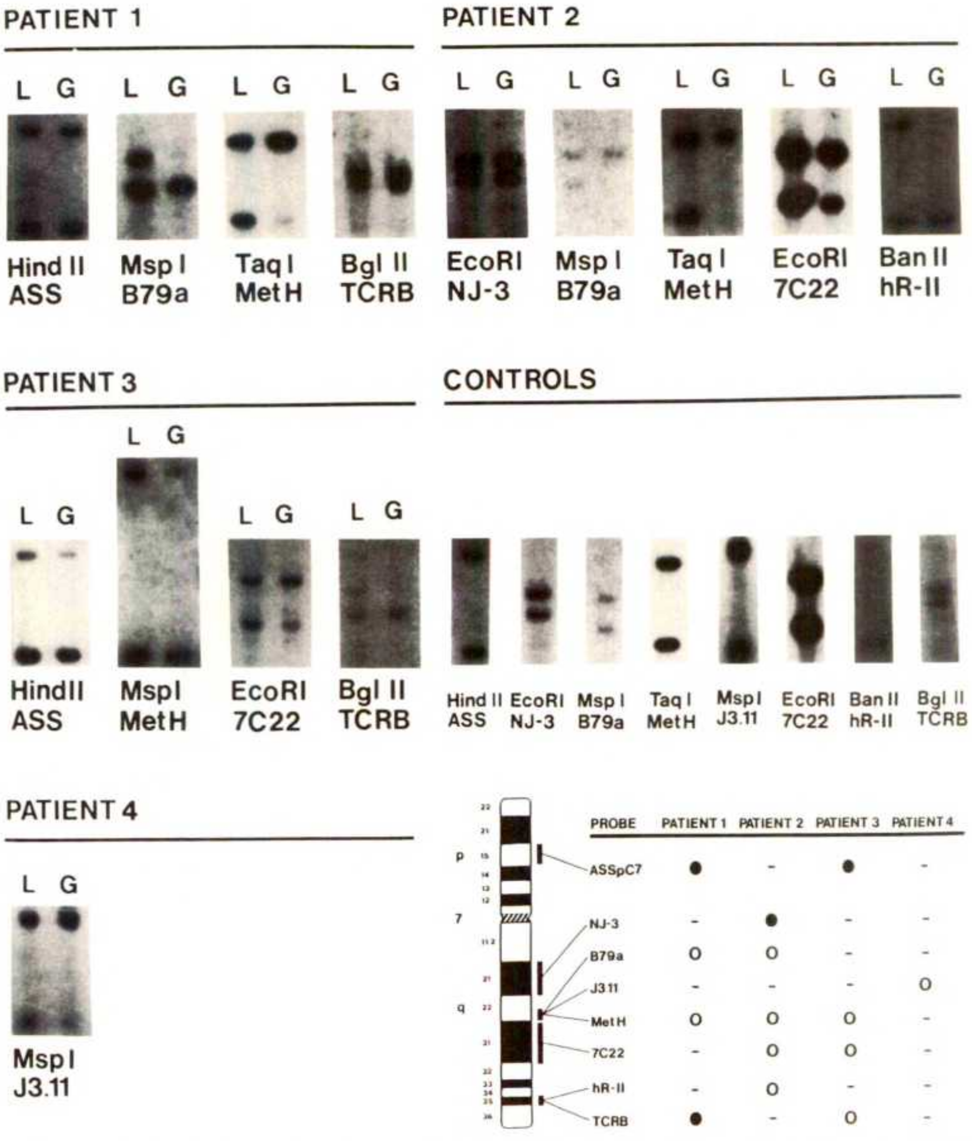


Fig 1. Partial karyotypes showing G-banded chromosomes 7 from two bone marrow cells. Patients 1 through 4 have a partial deletion of the long arm of one homolog of chromosome 7. In patient 1, the deletion could be interstitial del(7)(pter → q21::q32-35 → qter); in patient 2, a different interstitial deletion is possible; in patients 3 and 4, the appearance is compatible with a terminal deletion del(7)(q22). Patients 5 through 8 have two structurally normal chromosomes no 7.



PROBE	PATIENT 1	PATIENT 2	PATIENT 3	PATIENT 4
ASSpC7	●	-	●	-
NJ-3	-	●	-	-
B79a	O	O	-	-
J3.11	-	-	-	O
Met H	O	O	O	-
7C22	-	O	O	-
hR-II	-	O	-	-
TCRB	●	-	O	-

Fig 2. Informative Southern blots of patients with a 7q− chromosome. DNA was extracted from separated blood lymphocytes (L) or granulocytes (G). Purity of the cell fractions was determined from cell preparations stained with May-Grünwald-Giemsa or Sudan black B: Patient 1: L 80%, G 99%; patient 2: L 91%, G 89%; patient 3: L 96%, G 71%; patient 4: L 95%, G 66%. The restriction enzyme and probe are shown under each panel. The sizes of the allelic bands are shown in Table 2. Lower right, a schematic representation of the presence of one or two allelic bands in granulocyte-derived DNA from patients 1 through 4. Absence or weakness of an allelic band (O); two allelic bands (●); patient not informative due to homozygosity (—) (Table 3).

Table 3. Constitutional Restriction Fragment Length Polymorphisms of Patients as Determined by Study of Lymphocyte-Derived DNA

Patient No.	Restriction Fragment Length Polymorphisms										
	ASSpC7 *HindII*	NJ-1 *MspI*	NJ-3 *EcoRI*	B79a *MspI*	MetH *MspI*	MetH *TaqI*	MetD *TaqI*	J3.11 *MspI*	7C22 *EcoRI*	hR-II *BanII*	TCRB *BglII*
1	1, 2	2	1	1, 2	2, 3	1, 2	1	2	1	2	1, 2
2	1	2	1, 2	1, 2	2, 3	1, 2	1	2	1, 2	1, 2	1
3	1, 2	2	1	2	1, 2	1	1	2	1, 2	2	1, 2
4	1	1	2	2	2	1	1	1, 2	1	2	2
5	ND	2	1	2	2	1	1, 2	2	1	ND	ND
6	ND	2	1, 2	1	1, 3	1, 2	1	2	1, 2	ND	ND
7	ND	2	1	2	2, 3	1, 2	1	2	1	ND	ND
8	ND	1, 2	2	1, 2	2	1	1,2	2	1	ND	ND

Numbers indicate alleles: 1 corresponds to larger fragment, 2 (or 3) to smaller fragment (fragment sizes given in Table 2). ND, not determined.

and without a deleted chromosome 7, which may be quite likely in patient 3 who had both cytogenetically normal and abnormal mitoses in the bone marrow (Table 1).

Acquired hemizygosity or homozygosity was not observed with various chromosome 7-specific markers in patients with two structurally normal chromosomes 7. This can be interpreted to suggest that molecular changes did not occur in chromosome 7 in these patients. Distinct molecular changes involving relevant genes may be present, however, but are not necessarily detectable with these probes. Two genes that regulate the function of hematopoietic tissue (erythropoietin and platelet-derived growth factor) have been mapped to chromosome 7[30,31] and others may still be undetected. The nature of the genes on chromosome 7 that may play a role in the development of MDS or ANLL is unknown at present.

Proximal breakpoint. The *MET* oncogene locus and the anonymous probes J3.11 and B79a have all been mapped to band 7q22.[21-23] These loci were deleted in all informative patients with a 7q− chromosome. Provided that the cytogenetic analysis correctly defines the breakpoint to 7q22, our results suggest that these probes are in the distal part of that band. Probe 7C22 was originally localized more distally (band q31) but closely linked with the cystic fibrosis locus.[24] In the granulocyte fraction of patients 2 and 3, however, one allelic band detected by probe 7C22 was weak but unexpectedly strong in comparison with the weaker allele detected by probes MetH or B79a. We have been unable to find a satisfactory explanation for this observation.

Probe NJ-3 detected two allelic bands of equal intensity in the lymphocyte and granulocyte fractions in patient 2 in whom deletion was detected with probes B79a, MetH, 7C22, and hR-II. The simplest interpretation is that the locus detected by probe NJ-3 is proximal of the other loci and not included in the deletion in this patient, which is in accordance with the published localization of the probes,[20-24] and with our cytogenetic interpretations (Table 1, Fig 1). All these loci are linked to the cystic fibrosis locus and map to a relatively short segment on the long arm of chromosome 7.[20-24]

Distal breakpoint. Assuming that not all deletions are terminal,[2] a distal breakpoint must also be defined. The T cell receptor β chain gene (*TCRB*) is located in band 7q35, and probe hR-II is closely linked to the *TCRB* locus.[25-27] Patients 1 and 3 were heterozygous for the TCRB polymorphism, and patient 2 was heterozygous for the hR-II polymorphism. In patient 3, one of the two allelic bands detected by the TCRB probe was very weak in the granulocyte derived sample. A similar finding was obtained with patient 2 using the hR-II probe. These results are compatible with the deletion extending distally of *TCRB* and *hR*-II, but do not necessarily imply a terminal deletion. Patient 1, however, showed two equally strong allelic bands with the TCRB probe in both lymphocyte and granulocyte fractions. The most simple interpretation is that an interstitial segment of the chromosome has been deleted and the terminal part including *TCRB* has been retained in patient 1. These results are in agreement with the cytogenetic results that show an interstitial deletion. We conclude that the 7q− chromosomes were different in terms of deleted genomic material in different patients and that interstitial deletions exist.

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Shear Stress Activation of Platelet Glycoprotein IIb/IIIa Plus von Willebrand Factor Causes Aggregation: Filter Blockage and the Long Bleeding Time in von Willebrand's Disease

By J.R. O'Brien and G.P. Salmon

The article explores the finding that high shear alone applied to normal, native blood results in platelet aggregation. A filter with tortuous capillary-sized channels permits a study of the effect of shearing forces at different pressures. Native, heparinized, citrated and EDTA blood and platelet-rich plasma (PRP) were forced through the filter. Normal and von Willebrand's blood were studied, as were the effects of antibodies to platelet glycoproteins (GPr) and to von Willebrand's factor (vWf) and of "membrane-active" drugs. Normally, the filter blocked at 40 mmHg but not at 5 mmHg. Transmission electronmicroscopy of the filter at 40 mmHg showed blockage by platelet aggregates. Initially, the mean transit time through the filter was 8 milliseconds. Platelet retention in the filter occurred in two phases. From 0 to 3 seconds, only high-shear, vWf, and GPrIIb/IIIa were required. From 10 to 20 seconds, retention presumably involved these three attributes, but divalent cations were also essential. Only this phase was inhibited by some membrane-active drugs. ADP- and thrombin-induced aggregation requires GPrIIb/IIIa and fibrinogen. Shear-induced blocking of the filter by blood with a normal concentration of fibrinogen requires GPrIIb/IIIa and vWf. This indicates a different type of exposure of GPrIIb/IIIa. The long bleeding time in vW disease highlights the absolute requirement for vWf and emphasizes the difference in exposure of GPrIIb/IIIa induced by shear stress. Evidently, a process similar to that occurring in the filter is required in normal capillary hemostasis.

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THE LONG TEMPLATE bleeding time at 40 mmHg in von Willebrand's disease (vWd) is not fully understood. Ristocetin apart, vW platelets behave normally in most tests, but abnormalities are regularly reported in conditions of high shear.[1-8] This article explores the paradox that with high shearing forces, when it must be difficult to secure hemostasis, von Willebrand's factor (vWf) is required. At low pressure, evidently other mechanisms are involved.

If native blood is forced through a polycarbonate Nuclepore filter with 5-μm holes, only a few single platelets adhere to the upper surface. Thus, the material is relatively inert. On the underside, massive clumps of platelets are deposited.[9,10] Sheared platelets will aggregate and block small holes[11,12] and filters.[13-15] A thin filter with tortuous capillary-sized channels permitted a detailed study of the effects of the shearing forces necessary to produce platelet aggregates that are retained and block the filter.

MATERIALS AND METHODS

The device is shown diagrammatically in Fig 1. The commercial filter is made of glass fibers 0.1 to 3.4 μm in diameter; they run in all directions (Fig 2), creating tortuous channels of varying geometry through the filter. Particles >10 μm in diameter are retained. The filter, effectively 9 mm in diameter, is held in a filter holder (Nuclepore, Pleasanton, CA). The basic method is as follows. Modifications are outlined in the Results section. First, with tap Y closed, the air reservoir is filled to the required pressure through X. The blood syringe, a 2-mL (sometimes a 5-mL) routine plastic syringe is modified by passing a 16-gauge butterfly needle through the rubber flange of the plunger, which remains in the fully extended position. Blood, 2 or 5 mL, is aspirated into this syringe by suction, with another syringe attached to C in tap Y. A loaded filter holder is attached to the blood syringe, and the assembly is clamped over the drop counter. A is connected to B, and the blood is forced through the filter. The first effluent drop triggers a timer and drop counter, and the cumulative drops at 5-second intervals are recorded manually. The effluent drops during the first 3 seconds, and those emerging between 10 and 20 seconds are collected into EDTA tubes.

The cumulative drop count can be plotted against time (Fig 3). A measure of the progressive rate of slowing—the "drop ratio"—is obtained by dividing the average number of drops/5 sec recorded between 10 and 20 seconds by the initial rate [(drops 10 to 20/2)/drops 0 to 5] × 100. Thus, a high ratio indicates little slowing.

Platelets and WBCs are then counted in the initial blood or platelet-rich plasma (PRP), in the 0- to 3- and the 10- to 20-second samples. From these, the percentage of cells retained is calculated and, since the the volume of blood at blockage is known (20 drops = 1 mL), the absolute number of platelets required to block the filter can be calculated.

The standard procedure, unless otherwise specified, was as follows. Nine milliliters of blood, collected by a perfect venipuncture, were delivered into a polystyrene/polypropylene tube (no. Z10PS/NL, Sterilin, Feltham, Middlesex, England) in which was 1 mL saline containing 40 IU heparin (Weddel, Wrexham, England). Aliquots tested within 2 hours gave results similar to those tested immediately provided that the blood was stored at room temperature and thoroughly mixed for 5 minutes to eliminate RBC rouleaux formation. The capped tubes contained 2 mL air, so that any pH change was small and constant. The pressure normally was 40 mmHg, and observations were continued until all the blood had run through or until the filter blocked, which is defined as ≤1 drop/5 sec.

An unused filter was visualized by scanning electronmicroscopy (SEM) (Fig 2). For transmission electronmicroscopy of the filters after passage of blood or PRP, the filtration was stopped after a predetermined time; the filter was removed and plunged within 10 seconds into 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate, postfixed in osmium tetroxide, dehydrated, and embedded in Spurr's resin. Despite the glass fibers, routine sections were satisfactory and were stained with uranyl acetate and Reynold's lead acetate.

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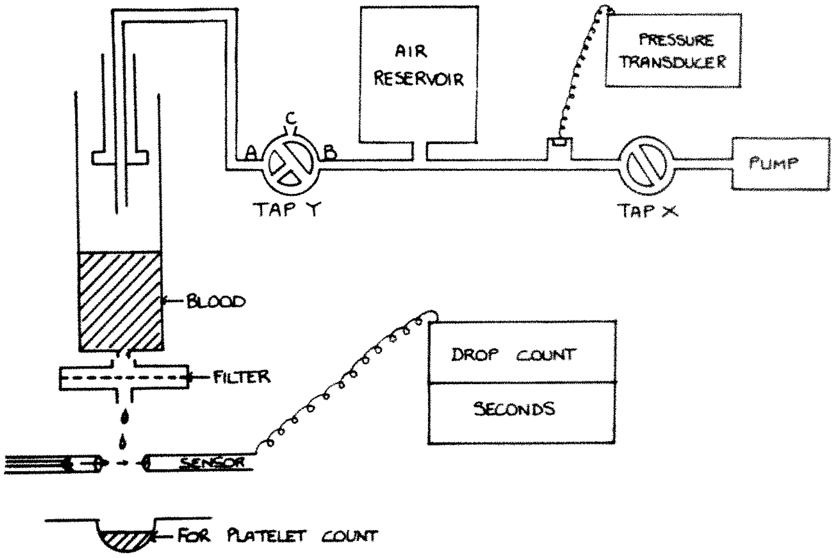

Fig 1. Diagrammatic representation of the device.

Chemicals and Antibodies

The following chemicals were obtained from Sigma, Poole, England: aspirin, apyrase (grade VIII), chlorpromazine, and chloroquine. Mepacrine was obtained from Boots, Nottingham, England, and cocaine was obtained from May and Baker, Dagenham, England. Stypven (Russell viper venom) and prostacyclin (PGI_2) were kindly presented by the Wellcome Foundation (Dartford and Beckenham, England). The antibodies to platelets GPrIb and IIb/IIIa were kindly supplied by Drs B.S. Coller and W. Nichols and the antibodies to vWf were supplied by Dr E.J.W. Bowie. The kit for the platelet factor 4 assay was obtained from Abbott Diagnostics, Wokingham, Berks, England. Cryoprecipitate was obtained from the National Blood Transfusion Service. It was selected to have no red cell agglutinins, warmed to 37°C, passed through a standard filter in the apparatus to remove any particles, and then added to blood warmed to 37°C.

Patients

Twenty patients with severe atherosclerosis were studied seven times in 3 months. The correlations presented are derived from comparisons of the mean value for each individual. (Less complete data on normal subjects were similar).

Sixteen vWd patients were repeatedly examined. They were considered to be types I, IIA, and B, with factor VIIIC, factor VIIIcAg, and RiCof from 0% to 40%. Multimeric analysis was not performed.

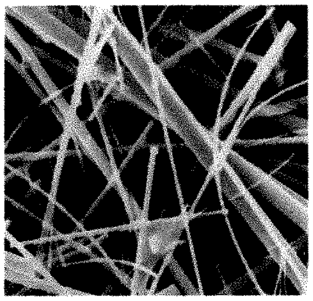

Fig 2. Scanning electronmicrograph of filter (original magnification ×1,800; current magnification ×810).

Statistics

The degree of correlation was measured by the product moment correlation coefficient (r). Reproducibility: the relative variability of samples was compared using Karl Pearson's coefficient of variation (CV).

RESULTS

Reproducibility

Within sample. Four aliquots from each of six people were tested at 40 mmHg. The mean CV of the drop count between 10 to 20 seconds was 9% ± 4%.

Within patient. Twenty patients with atherosclerosis were studied seven times over a 4-month period. The mean CV between these seven samples (10 to 20 seconds) was 20% ± 11%.

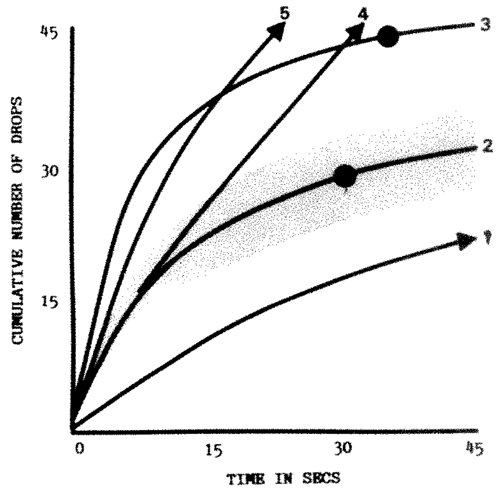

Fig 3. Cumulative drop count plotted against time. (1) Normal heparinized blood at 5 mmHg pressure. (2) Normal heparinized blood at 40 mmHg; shaded area indicates range (1 SD). (3) Normal heparinized PRP at 40 mmHg. (4) EDTA blood at 40 mmHg. (5) von Willebrands' blood. No blockage, (→); Filter "blocked" (●).

Between patients. The CV was established by comparing the mean of seven samples from each of the 20 patients: CV = 23%.

Platelet retention (10 to 20 seconds) figures were closely correlated with drop counts and had similar CVs.

Pressure

The crucial influence of different pressures was investigated first (Fig 3). Aliquots of the same heparinized normal blood were forced through the filter at different pressures. At 40 mmHg, blood initially passed rapidly through (14 ± 2 drops in 5 seconds; n = 40); within 20 seconds, however, marked slowing had occurred and on the average, the filter blocked in 34 ± 12 seconds (n = 40) when 214 × 10^6 ± 62 (n = 40) platelets had been retained.

At 100 mmHg, initially on the average, 16 drops were forced through the filter but the tracing, platelet retention, and blocking were similar to those at 40 mmHg. At 5 mmHg (Fig 3), the initial rate was slow, but little decrease in the rate occurred (drop ratio 62 ± 20) and no blocking occurred in the filter.

Rate of Passage Through Filter

The volume within the filter was estimated by two methods. The filters were filled with water, and the increase in weight was 8 mg. The overall dimensions (9 mm in diameter; 0.26 mm thick) together with the makers' claim that one-half the volume was glass, gave a calculated volume of 8 mm^3. Two milliliters EDTA PRP and vW PRP at 40 mmHg ran through in a continuous stream, and both took 2.1 seconds (n = 6). From this, the mean transit time can be calculated as 8 milliseconds. Normal heparinized PRP blocks the filter in 26 ± 7 seconds (n = 7). Before any platelets cause slowing, the transit time of normal PRP must be similar to that of EDTA or vW PRP. Thus, platelets exposed for 8 milliseconds to these shearing forces aggregate, accumulate, and finally block the filter.

Platelet Aggregation and Filter Blocking

Sections of the filter were prepared for TEM after 2, 8, and 20 seconds of flow, using normal blood or PRP at 5, 40, and 100 mmHg. Blood and PRP from vW patients, which do not block the filter, were also studied. At 40 mmHg, with normal PRP from 3 seconds on, increasing numbers of platelets were seen and the great majority of these had aggregated, with close apposition of the membranes (Fig 4). By 20 seconds, most of the filter was filled with a mass of aggregated platelets and some centering of the granules had occurred. Some filter fibers were surrounded by platelets, but throughout this time period naked fibers were seen with platelets close to them but with no apparent adhesion. Similarly, there was no apparent interaction of the few WBCs retained with the glass fibers or with platelets. Sections of normal blood at 40 mmHg were more complex, with many RBCs present; again, however, similar masses of platelet aggregates were seen.

At 100 mmHg, the findings were essentially similar to those at 40 mmHg. At 5 mmHg, with normal PRP fixed at the same times, far fewer platelets were present than at 40 mmHg. Although there were some sites of close parallel apposition of platelet membranes (aggregation), more free platelets were apparent. Again there was no evidence of active adhesion to the glass fibers. The appearances using vWd PRP at 40 mmHg were similar to those of normal PRP at 5 mmHg: Few platelets were retained and little aggregation was seen (Fig 5).

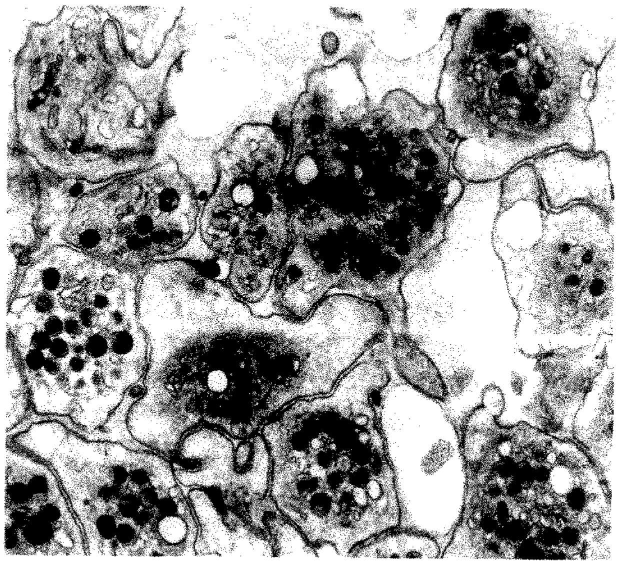

Fig 4. Normal heparinized PRP passed through this filter at 40 mmHg for 8 seconds and fixed within 10 seconds. Many platelets are retained, and already extensive close parallel appositon (aggregation) and early centering of the granules is apparent. Original magnification ×29,160; current magnification ×14,000.

Cellular Damage

Cell damage could lead to leakage of aggregating agents from inside the cells. Effluent normal blood was centrifuged; the degree of hemolysis varied form 0 to, on occasions, 0.25%, but this did not correlate with blockage (n = 20). The plasma platelet factor 4 before passage was 10.6 ng/mL; after passage, the factor 4 was 26 ng/mL (n = 3). This increase would occur if 0.2% of platelets were lysed. The Stypven time of native blood showed little change (35 seconds before and 33 seconds after passage; n = 2). Thus, mechanical damage was minimal.

Calcium and Heparin

An absolute requirement for divalent cations was shown as follows. When native blood was tested at once, blocking occurred in 20 ± 6 seconds (n = 12). Aliquots (2.5-mL) of the same blood collected into citrate (final concentration 0.38%) did not block the filter but ran through in 33 ± 8 seconds (n = 5). Aliquots collected into liquid EDTA (final concentration 0.4%) poured through even faster (22 ± 7 seconds (n = 8)), and few platelets were retained (Fig 3, Table 1).

Blood collected into heparin (final concentration 4.0 IU/mL) blocked the filter in 34 ± 12 seconds (n = 40). Heparin, 20 IU/mL gave similar results.

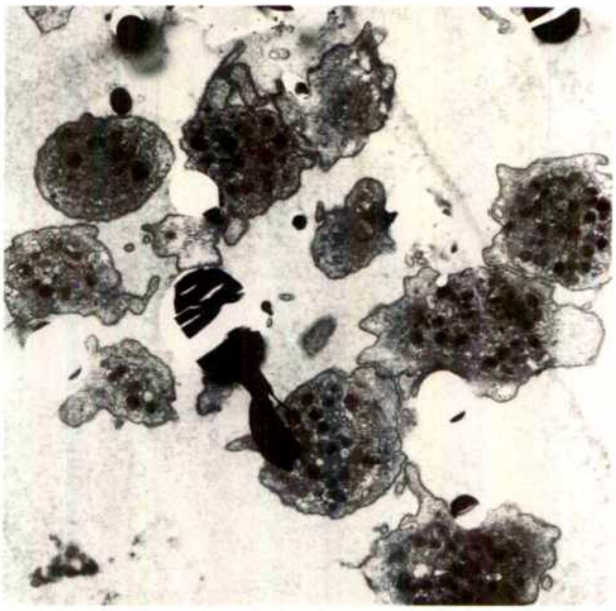

Fig 5. **Heparinized PRP from a patient with vWd was passed at 40 mmHg through the filter for 8 seconds and fixed within 10 seconds. Few platelets were retained. Few areas of close parallel platelet membrane apposition–aggregation are evident. The black objects are transected and shattered glass fibers. Original magnification ×29,160; current magnification ×15,450.**

Effect of RBCs and WBCs

Normal heparinized PRP contained very few RBCs and few WBCs (0.3 to 1.2 × 10^9/L) and, when tested at 40 mmHg, the PRP initially poured through in a continuous stream; slowing (bending in the tracing is shown in Fig 3) was rapid, however, and blocking occurred in 26 ± 7 seconds (n = 7). Even when the pressure was decreased until the initial drop rate of the PRP was similar to that of blood, eg, 14 drops/5 sec, blocking still occurred at 35 ± 5 seconds (n = 3).

WBCs probably do not contribute to filter blocking because PRP with a WBC count of 0.3 to 1.5 × 10^9/L blocked the filter more quickly than did the whole blood with a WBC count of 3 to 10 × 10^9/L. In patients with WBC counts up to 17 × 10^9/L results were normal. WBC retention was initially 45 ± 10% (n = 40) and similar to platelet retention (50% ± 12%), but between 10 and 20 seconds the WBC retention decreased to 22% ± 11% while the platelet retention increased (66% ± 15%).

Table 1. Platelet Retention and Drop Ratio of Normal, vWf, and EDTA Blood at 40 mmHg

Blood Type	n	Retention (%) 0-3 s	Retention (%) 10-20 s	Drop Ratio
Normal hep blood	40	50 ± 12	66 ± 15	35 ± 11
Normal hep blood + cryo	8	78 ± 2	92 ± 2	30 ± 9
vW hep blood	16	28 ± 15	24 ± 16*	53 ± 7
vW hep blood + cryo	8	61 ± 22	83 ± 8	18 ± 5
Normal EDTA blood	8	50 ± 8	13 ± 5*	61 ± 11

vW, von Willebrand's disease; hep blood, blood collected into heparin 4 IU/mL; + cryo, 0.5 mL cryoprecipitate added to 2.0 mL hep blood; EDTA blood, blood collected into 0.4% EDTA

*Filter did not block; others blocked in <40 seconds.

The initial hemoglobin and the WBC count of blood correlated with, and so probably influenced, the initial drop rate ($r = -.35$ and $-.41$, respectively; n = 20), but they had no effect on platelet retention. The percentage of platelets retained in 0 to 3 seconds correlate with the percentage retained between 10 and 20 seconds (r = .82; n = 20). WBC retention 0 to 3 seconds did not, however, correlate with any other parameters; on the other hand, WBC retention (10 to 20 seconds), although low, tightly correlated with the platelet retention (10 to 20 seconds) ($r = .70$; n = 20).

vWf

Neither heparinized nor native blood from 16 patients blocked the filter at 40 mmHg; 2.5 mL blood ran through in 17 ± 7 seconds with little slowing (the drop ratio was 53 ± 7, and platelet and white cell retention was low) (Fig 3, Table 1). Even at 100 mmHg, there was equally little slowing; 2.5 mL ran through in 17 ± 8 seconds (n = 12).

Cryoprecipitate, 0.5 mL, added to 2 mL vWf blood raised the concentration of vWf from 27% ± 8% to 200% ± 6% (n = 5). Normal blocking occurred rapidly, with normal platelet (Table 1) and WBC retention.

Inhibition of Blocking (Table 2)

Inhibition of normal platelet retention and filter blocking was explored (a) by altering the temperature, since most enzymes are temperature-dependent; (b) by altering specific enzyme pathways; (c) by using specific antibodies to cover receptor sites on the platelets; and finally (d) by using nonspecific membrane-active drugs. All inhibitors were added 1 minute before testing.

Temperature. Heparinized blood collected in a room at 37°C and tested at once gave results similar to those obtained with aliquots incubated at room temperature for ≤ 1 hour (37°C: 33 ± 3 drops at 30 seconds; 22°C: 29 ± 4; n = 6).

Aspirin. When added in vitro, aspirin (10 mmol/L final concentration in distilled water) had no significant effect (Table 2). When patients swallowed 300 mg aspirin 2 hours before venepuncture, the filter results were similar to the pre-aspirin tests (n = 3).

Prostacyclin. At high concentration, prostacyclin (dissolved in 50 mmol/L Tris HCl buffer, pH 9.5) had no effect on platelet retention at 0 to 3 seconds, but significantly inhibited platelet but not WBC retention between 10 and 20 seconds. The addition of this buffer alone had no effect.

Apyrase. In the aggregometer, apyrase 1.7 U/mL, dissolved in distilled water abolished aggregation induced by ADP mol^{-4}/L. In the filter, the addition of mol^{-4}/L to heparinized normal blood at 37°C 1 minute before testing decreased the blockage time from 30 to 15 seconds (n = 3). The addition of apyrase before the ADP completely abolished the ADP effect. Thus, the apyrase was fully active. Added to normal heparinized blood, it had no effect on the filter results (n = 6).

Chloroquine. At a final concentration of 0.15 mmol/L this drug, dissolved in distilled water, consistently promoted

Table 2. Platelet and WBC Retention and Drop Ratio in the Presence of Inhibitors

Addition	Concentration	n	Platelet Retention 0-3 s	Platelet Retention 10-20 s	Drop Ratio	WBC Retention 0-3 s	WBC Retention 10-20 s
Chlorpromazine	1.7 mmol/L	4	95	25*	172*	124	63
Cocaine	2.9 mmol/L	7	109	54*	156*	105	64
Chloroquine	1.5 mmol/L	4	108	29*	153*	123	117
Mepacrine	1.0 mmol/L	4	86	26*	157*	131	130
PGI_2	25 ng/mL	5	119	51*	157*	113	127
Aspirin	10 mmol/L	4	—	91	108	—	100
Apyrase	1.7 U/mL	5	125	114	78	88	100

Results are expressed as mean percentage relative to various control results. A low retention figure and a high drop ratio figure indicate inhibition.
*$p < 0.01$

rapid blocking and platelet retention increased from 65% to 91% (n = 3). At a concentration of 0.38 mmol/L, no effect was noted, but at 1.5 mmol/L platelet retention between 10 and 20 seconds was reduced to 30% of the control values and no blocking occurred.

Chlorpromazine, mepacrine, and cocaine. Relatively high concentrations of chlorpromazine, mepacrine, and cocaine dissolved in distilled water produced gross inhibition between 10 and 20 seconds, with results similar to chloroquine 1.5 mmol/L. The effect of lower concentrations was not explored.

Inhibition of WBC retention (Table 2). None of the inhibitors affected WBC retention between 0 and 3 seconds, but on every occasion between 10 and 20 seconds WBC retention was decreased by chlorpromazine and cocaine. Chloroquine, mepacrine, and PGI_2 had no effect.

Effect of antibodies to GPrIb, GPrIIb/IIIa, and vWf (Table 3). Three antibodies to GPrIIb/IIIa were available; one to GPrIb was available. These were reconstituted in distilled water so that 0.1 mL added to 5 mL of normal, heparinized blood gave final concentratoins of 5 to 13 µg/mL. These mixtures were incubated at room temperature for 1 minute. Controls consisted of 5-mL aliquots treated indentically but with no additions. The order of testing was varied. The results expressed as a percentage of the related controls are given in Table 3. All three antibodies to GPrIIb/IIIa halved the platelet retention between 0 and 3 seconds, and two reduced retention 10 to 20 seconds to 20% and 22% of the control values. The third antibody, 7E3, was less active, but with all three antibodies there was little progressive decrease in the drop rate, ie, a high drop ratio. All three also inhibited WBC retention during the period of 0 to 3 and 10 to 20 seconds.

Table 3. Platelet Retention and Drop Ratio in Presence of Antibodies

Antibody Type	n	Platelet Retention 0-3 s	Platelet Retention 10-20 s	Drop Ratio
Antibodies to GPrIIb/IIIa				
10 E5[16]	3	49	22	139
HP1/1D[17]	4	61	20	121
7 E3[16]	2	53	54	111
Antibody to GPrIb				
6 DI[18]	4	105	80	124
Antibody to vWf				
W1-3[19]	5	92	55	145
W1-5[19]	5	68	34	182
93 E 4[19]	5	64	50	154
94 H 5[19]	5	80	53	150

Results are expressed as the mean percentage of the various control values.

The antibody 6DI at a concentration of 2.7 µg/mL completely inhibited ristocetin (1.5 mg/mL)-induced aggregation of normal PRP. Thus, it was fully active. Mixed at a concentration of 13 µg/mL for 1 minute with blood at 20°C, it had at most a minor effect (Table 3).

Four antiboides to vWf were each studied five times. Fifty microliters of antibody added 1 minute before testing to 5 mL normal heparinized blood inhibited blocking and, on average, platelet retention 0 to 3 seconds and 10 to 20 seconds were decreased respectively by 24% and 45% of the controls (Table 3).

DISCUSSION

The main conclusions from these experiments are that the filter is blocked by platelets in two phases. The first, lasting 0 to 3 seconds, requires shear stress, vWf, and GPrIIb/IIIa. The second (10 to 20 seconds) has an absolute requirement for divalent cations. Stress, vWf, and GPrIIb/IIIa are apparently still required.

A global test in which whole blood is forced through capillary-sized holes was deliberately chosen to simulate, in part, the in vivo bleeding time, and it is unlikely to be influenced by a single mechanism. Because Apyrase and aspirin have no significant effect, ADP and thromboxane A_2 are apparently not involved. Cyclic AMP may be (PGI_2), but three phosphodiesterase inhibitors (theophylline, aminophylline, and PGE_1: data not given) had no effect. Heparin and native blood results are similar, thus clotting and a heparin–platelet effect seem unlikely.[20]

This is the first experimental situation that has allowed a precise estimate of the speed of shear-induced platelet aggregation.[21,22] The initial mean transit time with normal PRP, and before aggregates obstruct the flow, is 8 milliseconds, yet in this time platelets are activated and aggregate. Subsequently, the filter is blocked when sufficient platelets have been retained, eg, 70% of platelets in 20 drops (1 mL blood) $\simeq$ 150 × 10^6 platelets.

The contribution of glass that can activate platelets, WBCs, and plasma seems excluded by the reported Nuclepore experiments.[9,10] From the evidence presented, it appears unlikely that WBCs initiate blockage. Chlorpromazine and cocaine inhibit WBC retention and chloroquine and mepacrine do not, but all four agents inhibit platelet retention (Table 2).

Although RBCs have a profound effect on the initial flow rate, their removal in PRP does not impair filter blocking. Thus, ADP leaking from even intact RBCs is irrelevant.

Numerous authors report that platelet retention in glass bead columns[24,25] and in vascular models is abnormal in vWf disease but only in situations of high shear.[26,27] The absence of filter blocking in this model in vWd, its correction by the additon of cryoprecipitate, and the correction by DDAVP[28-30] and the probable effect of high-mol-wt multimers of vWf[3,27] all support the concept that vWf is essential in conditions of high shear.

With use of monoclonal antibodies, it has been shown that GPrIIb/IIIa on the platelet membrane is necessary for filter blockage (Table 3) and that GPrIb is of little or no importance although it may be in other systems.[1,2] In purified systems, GPrIIb/IIIa has been reported to be activated, eg, by thrombin, at a site exposed with a conformational change[31,32] before vWf can bind to the platelet.[33] In these circumstances, however, if fibrinogen is present or added later, the fibrinogen will preferentially bind to the platelet surface or displace the vWf.[34,35] In the filter and in Baumgartner's models, normal fibrinogen levels are present, yet vWf is required. It follows that in stress-induced activation the expression of GPrIIb/IIIa must differ from that which occurs when aggregating agents such as ADP and thrombin are added. In the aggregometer, vWf platelets behave normally after the addition of ADP, and the GPrIIb/IIIa binds to the fibrinogen as the ligand.[16,36] In the high-shear situation, both adhesive proteins, vWf and fibrinogen, may be required,[37] but this does not alter the conclusion that high shear causes a unique expression of GPrIIb/IIIa that requires vWf for platelet aggregation.

The differences in platelet retention between 0 to 3 seconds and 10 to 20 seconds show clearly that two processes are involved, although the events at 0 to 3 seconds (first phase) must also contribute to or even determine the events in the second phase (10 to 20 seconds) since they are closely correlated. There are important differences, however. Table 4 shows that phase 1 requires high shear, vWf together with "available" platelet membrane GPrIIb/IIIa, but is independent of divalent cations (EDTA blood). Phase 2 is presumably also dependent on vWf and GPrIIb/IIIa but differs from phase 1 in that it is inhibited in the absence of divalent cations and by the addition of membrane stabilising drugs.

Table 4. Differences Between Platelet Retention During First Phase (0-3 s) and Second (10-20 s)

Addition	n	Platelet Retention 0-3 s	Platelet Retention 10-20 s
Nil (normal)	40	100	100
vW	16	56	36
Anti-GPrIIb/IIIa	9	54	32
EDTA	8	100	20
Membrane-active drugs	19	100	34
PGI_2	5	119	51

Average differences in platelet retention are expressed as a percentage of the appropriate normal values.

Shear stress initially appears to expose GPrIIb/IIIa in a way that is independent of fibrinogen and divalent cations. This locus with vWf permits platelet aggregation. Thereafter, the same or another vWf molecule becomes attached to GPrIIb/IIIa through a cation-dependent bond, and only this second step is inhibited by perhaps a more general membrane perturbation caused by membrane-active drugs.

The effect of these membrane-active drugs, in admittedly high (mmol/L) concentrations, is not fully understood. The stimulating effect of lower concentrations of chloroquine is typical of some membrane-active drugs. Cocaine and quinine inhibit ADP- and thromobin-induced aggregation.[38] Inhibition by local anesthetics of transmembrane calcium migration[39] and the inhibition of filter blocking by EDTA may be related phenomena. Dibucaine and chlorpromazine are reported to influence membrane phospholipase A2.[40] The inhibitory effect of PGI_2 at 25 ng/mL may be due to a "membrane-stabilizing" effect,[41] and PGI_2 is reported to prevent the binding of vWf to platelet GPrIb.[42] The lack of temperature dependence favors a conformational change rather than an enzyme effect. Finally, it must be pertinent that apyrase, PGE_1 and dibucaine all inhibit the rate of binding of antibody to GPrIIb/IIIa in both buffer- and ADP-stimulated platelets.[31]

In conclusion, we propose, in agreement with Schmid-Schonbein et al[43] that acute rapid bending of the platelet causes membrane perturbation and that this alone can, in milliseconds, induce a unique exposure of GPrIIb/IIIa. Initially, only vWf is required. Seconds later, calcium is required for further platelet aggregation. Collagen, subintima,[44] RBCs,[45] and thrombin are not required for this type of aggregation, nor probably is fibrinogen[46] or ADP. This is the first clear demonstration of a further independent way of inducing platlet aggregation. Accordingly, in patients with vWd, the long bleeding time at 40 mmHg results from an absence of vWf, which is essential for shear-induced platelet aggregation in this system and presumably in other systems. In contrast, ADP and thrombin involve a different expression of GPrIIb/IIIa[34] since aggregation then requires fibrinogen[31,34,37] and vWf is irrelevant. Thus, ADP and thrombin apparently do not play a major part in determining the normal bleeding time. These findings presumably are relevant to hemostasis and thrombosis.

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von Willebrand Factor Is Present on the Surface of Platelets Stimulated in Plasma by ADP

By Burt Adelman, Patricia Carlson, and Patrick Powers

von Willebrand factor (vWf) can bind to glycoprotein (GP) IIb/IIIa on activated platelets. The significance of this interaction is unclear, however, because it has not been possible to detect vWf binding to GPIIb/IIIa on platelets stimulated in plasma. We have developed an indirect, flow cytometry assay that uses fluorescein-labeled antibodies to detect vWf and fibrinogen on platelets. Using this assay, we found vWf on the surface of platelets stimulated in plasma by ADP. The number of platelets that bound vWf increased in proportion to ADP concentration and incubation time. Washed platelets in a protein-free buffer activated by 1 μmol/L calcium ionophore A23187 or 10 μmol/L ADP also bound vWf, suggesting that we were detecting surface binding of α-granule–derived vWf. Monoclonal antibodies against the vWf binding site on GPIb (6D1) and the vWf and fibrinogen binding sites on GPIIb/IIIa (LJP5 and LJ-CP8, respectively) were used to characterize the mechanism of vWf binding to stimulated platelets. Ristocetin-induced binding of vWf was inhibited by 6D1, and ADP-induced binding of fibrinogen was inhibited by LJ-CP8. None of these antibodies inhibited ADP-induced vWf binding. Aspirin and prostaglandin E_1 also inhibited ADP-induced binding of vWf in platelet-rich plasma. During platelet activation in plasma, vWf derived from α-granules becomes bound to the platelet surface possibly being transferred already associated with a binding site.

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PLATELET ADHESION and aggregation are the first hemostatic events to occur following vascular injury. These processes depend on platelet association with the adhesive proteins von Willebrand factor (vWf) and fibrinogen. Although much is known about these initial steps of normal hemostasis, the exact molecular mechanism of platelet plug formation has not yet been defined. In vitro in the presence of ristocetin, von Willebrand factor binds to the platelet membrane glycoprotein Ib (GPIb).[1] Von Willebrand factor binding to GPIb is not energy dependent and will occur even if platelets have been formaldehyde-fixed.[2] In contrast, fibrinogen binding to the GPIIb/IIIa complex is energy dependent and occurs only after platelet activation.[3] The current model of platelet plug formation postulates that vWf–GPIb interaction is a passive process that mediates platelet adhesion to the subendothelium, after which platelet activation results in fibrinogen binding to GPIIb/IIIa and platelet aggregate formation.

Recent studies demonstrated that exogenous vWf can bind to GPIIb/IIIa on washed platelets stimulated by adenosine diphosphate (ADP) or thrombin.[4-11] Although Lombardo et al concluded that vWf and fibrinogen occupy distinct binding sites on GPIIb/IIIa,[6] the physiologic significance of exogenous vWf binding to GPIIb/IIIa has been questioned because it can be blocked by fibrinogen if present at the concentrations found in plasma.[4,5,11] In addition, Schullek et al were unable to detect binding of vWf to ADP-stimulated platelets in platelet-rich plasma (PRP).[10]

Parker and others have demonstrated that α-granule–derived vWf can be detected on the surface of thrombin-stimulated washed platelets and that it is bound to GPIIb/IIIa.[4,6] To date, no investigations have been conducted that could detect the presence of secreted, platelet-derived vWf on the surface of platelets activated in plasma. Because platelet α-granules store vWf and their membranes contain GPIIb/IIIa,[12-14] we considered the possibility that activated platelets might transfer endogenous vWf directly to their surface by binding to α-granule GPIIb/IIIa. To examine this hypothesis, we developed a unique immunofluorescent flow cytometry assay to detect vWf on platelets stimulated in plasma by ADP. Our data suggest that vWf can be detected on the surface of these platelets and that it is derived from secreted α-granule stores.

MATERIALS AND METHODS

Normal human PRP and washed platelets were prepared from sodium-citrate anticoagulated blood as previously described.[15] Monospecific fluorescein-labeled goat anti-human vWf IgG antibody and fluorescein-labeled nonspecific goat IgG were obtained from Atlantic Antibodies (Scarborough, ME). Monospecific fluorescein-labeled goat anti-human fibrinogen IgG antibody ($F(ab')_2$ fragment) was obtained from Cooper Biomedical (Malvern, PA). The monoclonal antibodies LJ-CP8 (Fab fragment) and LJP5 (Fab fragment) were kindly provided by Dr Zaverio M. Ruggeri, Scripps Clinic and Research Foundation, La Jolla, CA. The monoclonal antibody 6D1 was kindly provided by Dr Barry Coller, SUNY, Stony Brook NY.[1,6] Aspirin and prostaglandin E_1 were obtained from Sigma Chemical, St Louis. All other materials were reagent grade or better and were obtained from common suppliers.

Assessment of vWf and fibrinogen binding to platelets in PRP by fluorescent flow cytometry. PRP was incubated at room temperature with 5 μmol/L ADP or 1.5 mg/mL ristocetin. The platelet–agonist mixture was swirled only once during incubation because constant mixing promotes formation of large aggregates, which interfere with subsequent analysis. The reaction was stopped by addition of an equal volume of buffered 2% formaldehyde. The platelets were formaldehyde fixed and washed as previously

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described[15] and immunostained with fluorescein-labeled anti-vWf or anti-fibrinogen antibody by incubation at 4°C for 30 minutes. The antibodies were used at saturating concentrations, which were determined by trial and error. After staining, excess antibody was removed by washing the platelets twice by centrifugation (1,200 g for 10 minutes 4°C) in phosphate-buffered saline (PBS) (0.01 mol/L sodium phosphate, 0.15 mol/L NaCl, pH 7.4). After the final wash, the platelets were diluted to ~50,000 per μL in filtered PBS. In some experiments, platelet activation was inhibited by the addition of aspirin and prostaglandin E_1 (final concentrations 2.5 mmol/L aspirin and 3 μmol/L PGE_1) to the PRP prior to ADP stimulation. In these experiments, the platelets were incubated with the inhibitor mixture for 15 minutes at 37°C prior to activation.

We used an Ortho 50H fluorescence flow cytometer equipped with a 2140 data handling computer (Ortho Diagnostics, Westwood, MA) and a 5-W argon-ion laser run at 250 mw power for fluorescence analysis of immunolabeled platelets. Calibration of the fluorescence gain settings was done daily using Fluorotrol GF (provided by Ortho Diagnostics). To prevent inclusion of aggregated platelets or platelet fragments, a sizing gate was set by examination of both right-angle and forward-angle scatter signals from formaldehyde-fixed resting platelets. Approximately 80% of resting platelets were included within this gate. Following activation by ADP or ristocetin, 65% ± 10% (SD) of all platelets were included within this gate. Fluorescence analysis of platelets that fell within the sizing gate was then conducted using log amplification. For each sample, 10,000 platelets were analyzed. Stimulated platelets labeled with anti-vWf or anti-fibrinogen antibody whose fluorescence signal intensity was greater than that of 100% of similarly stained resting platelets were counted as positive for agonist-induced binding of vWf or fibrinogen. Results are reported as percentage of positive platelets. Analysis of the data in this manner sacrifices sensitivity for specificity. By only counting as positive those cells whose fluorescence intensity is greater than 100% of the control cells we ignore all cells whose fluorescence intensity may have shifted to a higher level yet still remain within the control range.

Nonspecific binding of fluorescein-labeled antibody to resting and activated platelets was determined by conducting experiments identical to those described above except that fluorescein-labeled nonspecific goat IgG was substituted for specific antibody. The concentration of nonspecific antibody used was equivalent to that of the specific antibody. Less than 2.0% of all platelets in PRP activated by ristocetin or ADP bound more nonspecific goat IgG than did resting platelets.

Effect of anti-GPIIb/IIIa and anti-GPIb antibodies on vWf and fibrinogen binding to the surface of platelets stimulated in plasma. Monoclonal antibody 6D1 binds to platelet GPIb and blocks ristocetin-induced binding of vWf.[1] The monoclonal antibody LJP5 binds to the vWf binding site on GPIIb/IIIa and blocks ADP-induced binding of exogenous vWf to GPIIb/IIIa and when used as a Fab fragment does not interfere with fibrinogen binding to GPIIb/IIIa.[6] Monoclonal antibody LJ-CP8 also binds to GPIIb/IIIa and blocks binding of both fibrinogen and vWf to GPIIb/IIIa on activated platelets. This antibody was produced and characterized in the same fashion as other anti-GPIIb/IIIa antibodies,[6] (Z.M. Ruggeri, personal communication, March, 1987).

In studies identical to those described above, each antibody was independently added to PRP prior to platelet activation by 5 μmol/L ADP or 1.5 mg/mL ristocetin. The antibody was allowed to incubate with the PRP for 5 minutes prior to platelet activation. In each case, the antibody concentration was adequate to cause total inhibition of binding of fibrinogen or vWf to the targeted membrane receptor, either GPIIb/IIIa (LJP5 and LJ-CP8) or GPIb (6D1). The antibody concentrations used were determined both by trial and error and by review of previously published data.[1,6]

vWf expression on the surface of activated washed platelets. Washed platelets were prepared as previously described[16] and then suspended in TBS (0.01 mol/L Tris, 0.15 mol/L NaCl, pH 7.4) containing 4 mmol/L $CaCl_2$. The washed platelets were activated by addition of 1 μmol/L calcium ionophore A23187 or 10 μmol/L ADP, and the reaction was stopped at various intervals by formaldehyde fixation as described above. The ionophore was prepared as a 100 μmol/L stock solution in dimethyl sulfoxide (DMSO). After fixation, the platelets were immunostained with anti-vWf antibody and analyzed by flow cytometry. In control experiments, DMSO alone was substituted for ionophore.

RESULTS

We were able to detect vWf associated with the platelet surface after activation in PRP. As shown in Fig 1, the number of ADP-stimulated platelets with surface-bound vWf increased during the 5-minute incubation period. The number of platelets with surface-bound vWf also increased in relation to the concentration of ADP added; the peak effect was observed at 2.5 μmol/L ADP (Fig 2). The number of platelets with surface-associated vWf appeared even greater when the PRP was treated with ristocetin. After incubation for 5 minutes, 85% of platelets in PRP stimulated by 1.5 mg/mL ristocetin had surface-bound vWf as compared with 37% of platelets stimulated with 5 μmol/L ADP for the same time period (Fig 1). Mean fluorescence intensity of the entire platelet population also increased after stimulation, and the fluorescence histograms (fluorescence intensity *v* cell number) remained bell-shaped. Thus, platelets that bound vWf were not a unique subset of the entire population. The addition of aspirin and PGE_1 to PRP prior to ADP stimulation significantly reduced the number of stimulated platelets with surface-bound vWf (Table 1).

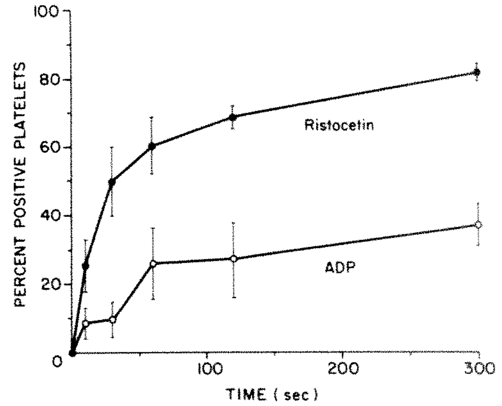

Fig 1. ADP- and ristocetin-induced expression of vWf by platelets in PRP. Five micromolar ADP (final concentration) or 1.5 mg/mL ristocetin was added to PRP. After incubation for the indicated time periods, the reaction was stopped by addition of buffered formaldehyde. Following fixation, the platelets were washed, immunostained with a fluorescein-labeled anti-vWf antibody, and prepared for analysis by fluorescence flow cytometry as described in the Materials and Methods section. Platelets with vWf on their surface were detected by their fluorescent signal and are indicated as the percentage of increase in positive platelets. For ristocetin activation, each point represents the mean (± SE) of separate determinations from four donors; forADP stimulation, each point represents the mean (± SE) of four or five separate determinations from four donors.

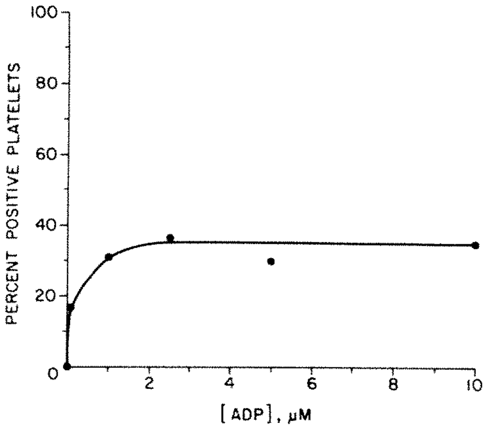

Fig 2. vWf expression on the surface of platelets stimulated by various concentrations of ADP. Platelets in PRP were incubated for 5 minutes with increasing concentrations of ADP (0.1 to 10 μmol/L). After activation, the platelets were prepared for analysis of vWf expression by fluorescent flow cytometry. The increase in percentage of positive platelets indicated a dose-related increase in vWf expression on platelets.

To demonstrate that our observations were not the result of nonspecific binding of fluorescein-labeled anti-vWf antibody to activated platelets, we examined the binding of fluorescein-labeled nonspecific goat IgG to ADP- or ristocetin-stimulated platelets. Platelet binding of nonspecific antibody did not increase with time following stimulation by either agonist and did not exceed the binding of specific antibody by resting platelets (data not shown). From these data, we concluded that our assay system detected the presence of vWf and fibrinogen associated with the platelet surface and not the nonspecific attachment of labeled antibody.

A number of investigators have demonstrated that endogenously secreted vWf can be found on the surface of activated platelets.[5,17-19] We therefore used our assay system to detect vWf derived from α-granules and associated with the platelet surface. Washed platelets in a protein-free buffer were activated by calcium ionophore or ADP. As shown in Fig 3, the number of platelets that expressed surface-bound vWf increased with time following addition of 1 μmol/L ionophore. Five minutes after activation, 50% of the platelets had detectable vWf on their surface. The number of platelets with vWf on their surface that were not ionophore stimulated never exceeded 5% of the total platelet population analyzed. Identical results were obtained when washed platelets were stimulated by ADP.

Table 1. Effect of Inhibition of Platelet Activation on ADP-Induced vWf Binding in PRP*

Incubation Period (s)	Percentage of Inhibition of vWf Binding Following Platelet Activation
10	100%
30	100%
60	89%
120	87%
300	73%

*PRP was incubated with 2.5 mmol/L aspirin and 4 μmol/L PGE$_1$ for 15 minutes at 37°C prior to addition of 5 μmol/L ADP. Each point represents the mean of two or three separate determinations.

Plasma vWf does not effectively compete with fibrinogen for binding to platelets activated in plasma; therefore, we believe that the vWf detected on platelets stimulated by ADP in PRP comes from α-granule stores. In addition, we hypothesize that vWf appears on the platelet surface already bound to an α-granule–derived receptor such as GPIIb/IIIa. To examine this possibility, we conducted a series of blocking experiments using monoclonal antibodies that bind to GPIb and block vWf binding (6D1), or bind to GPIIb/IIIa and block vWf (LJP5) and fibrinogen (LJ-CP8) binding[1,6] (Z.M. Ruggeri, personal communication, March, 1987). We used Fab fragments of LJP5 and LJ-CP8 to minimize nonspecific, stearic blocking effects. If our hypothesis was correct, exogenously added antireceptor antibody would not block vWf expression on ADP-stimulated platelets.

In separate experiments each antibody was added to platelet-rich plasma prior to platelet activation by ADP or ristocetin. Each antibody was used at a concentration that exceeded the minimum necessary to block binding of fibrinogen or vWf to the target membrane protein completely. Adding 6D1 to PRP completely inhibited the effect of ristocetin on expression of vWf but had no effect on vWf binding after platelet stimulation by ADP (Table 2). Fab fragments of the antibody CP8 totally inhibited fibrinogen binding to ADP-stimulated platelets but had no effect on ADP-induced vWf expression. Neither did the Fab fragments of the third antibody, LJP5, have any effect on ADP-induced binding of fibrinogen or vWf.

DISCUSSION

In this study, we demonstrated that vWf appears on the surface of platelets that have been stimulated by ADP in plasma. Although it is well established that vWf can bind to

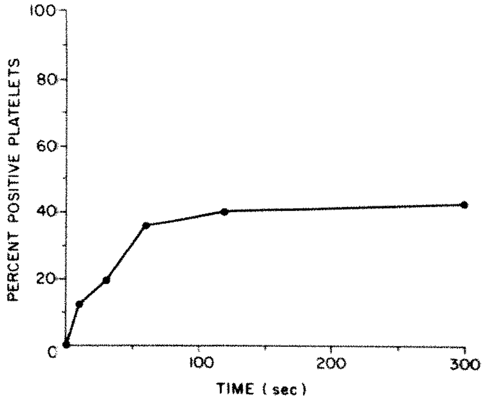

Fig 3. Calcium ionophore A23187-induced expression of vWf on the surface of washed platelets. Washed platelets in a protein-free buffer containing 4 mmol/L CaCl$_2$ were incubated with 1 μmol/L ionophore. At various time intervals, the incubation was stopped by addition of buffered formaldehyde and the platelets were prepared for analysis of vWf expression by fluorescence flow cytometry. Increased vWf expression is indicated by the rise in percentage of positive cells over time. Each point represents the mean of separate determinations from two donors.

Table 2. Effect of Anti-GPIb and Anti-GPIIb/IIIa Antibodies on vWf and Fibrinogen Binding to Platelets Stimulated in Plasma

Antibody* (Concentration)	Percentage of Inhibition of vWf or Fibrinogen Binding Following Platelet Activation		
	ADP		Ristocetin
	vWf (%)	Fibrinogen (%)	vWf (%)
6D1 (20 μg/mL)	0	—	100
LJ-CP8 (65, 130 μg/mL)	0	100	—
LJP5 (0.22, 0.45, 1.3 mg/mL)	0	0	0

Each antibody was added independently to PRP prior to addition of 5 μmol/L ADP or 1.5 mg/mL ristocetin (final concentrations). After incubation for 5 minutes, the platelets were formaldehyde fixed and assayed for vWf or fibrinogen expression as described in the Materials and Methods section. Each point represents the mean of two or three separate determinations.

*6D1 binds to GPIb and blocks vWf binding, LJ-CP8 binds to GPIIb/IIIa and blocks vWf and fibrinogen binding, and LJP5 binds to GPIIb/IIIa but blocks only vWf binding.

the GPIIb/IIIa complex after platelet activation,[4-8] no previous investigation has detected vWf on the surface of platelets activated by ADP in plasma. This is probably because fibrinogen, at the concentration found in blood, can totally block binding of exogenous vWf to GPIIb/IIIa even though there are distinct vWf and fibrinogen binding regions on the GPIIb/IIIa complex.[4-6,11] By using a fluorescein-labeled anti-vWf antibody to probe for platelet-bound vWf, we were able to detect the binding of α-granule–derived vWf to activated platelets. vWf from this source cannot be detected by conventional binding studies, which only follow platelet attachment of exogenously added vWf.

Platelet α-granules contain at least 10% of the total blood content of vWf, and, following platelet activation, secreted vWf is attached to the platelet surface.[4,17-19] Fernandez et al demonstrated that α-granules contain some vWf multimers that are larger than any normally found in plasma and that these molecules readily associate with the platelet membrane after platelet stimulation.[18] To confirm that our assay could detect surface-bound vWf derived from α-granules, we examined washed platelets stimulated by ionophore A23187 or ADP in a protein-free buffer. We found that stimulated washed platelets had vWf on their surface and that the percentage of platelets with surface-bound vWf increased with time. Nonactivated platelets did not demonstrate an increase in surface-associated vWf during the same time period.

We detected increased vWf on the surface of 37% of the ADP-stimulated platelets. This is probably an underestimate of the actual number of stimulated platelets with surface-bound vWf. Because we counted as positive only those cells whose fluorescence intensity was $>100\%$ of control cells, we probably excluded platelets that bound vWf but did not generate a fluorescence signal strong enough to be distinguished from the entire population of resting platelets. Such platelets, following stimulation, may have bound only enough vWf to cause their fluorescence intensity to shift from a lower to higher level within the control range. This possibility is supported by our observation that, following activation, the mean fluorescence intensity of the entire platelet population increased and the fluorescence intensity histogram remained bell-shaped. Setting too narrow a platelet-sizing gate on the flow cytometer may also have contributed to underestimating the number of vWf-positive platelets. Our gate was set to exclude all aggregates and particles smaller than 2 μm in diameter. A larger gate might have included more positive platelets but would have included particles and aggregates that might have introduced artifacts into the data. For the purpose of this study, we believe it appropriate to have used the most rigorous criteria for distinguishing positive from negative cells. Finally, although the conditions under which we conducted our studies, incubation at room temperature without stirring, may not permit optimal platelet release, these conditions were chosen because they match those of previous investigations of ADP-induced platelet attachment of vWf in plasma.[10]

Because plasma vWf cannot effectively compete with fibrinogen for binding sites on ADP-stimulated platelets, we hypothesized that the vWf detected on platelets stimulated in plasma was derived from α-granules and possibly transferred to platelet surface already bound to a membrane receptor. To investigate this hypothesis, we conducted a series of blocking studies using monoclonal antibodies directed against GPIb and GPIIb/IIIa. The antibody 6D1, a well-characterized anti-GPIb antibody,[1] completely blocked vWf binding to ristocetin-stimulated platelets in PRP but had no effect on platelet surface vWf association after ADP stimulation. Fibrinogen binding to ADP-stimulated platelets was blocked by Fab fragments of LJ-CP8, an antibody directed against GPIIb/IIIa that blocks both vWf and fibrinogen binding. Neither LJ-CP8 nor Fab fragments of LJP5, an antibody that specifically blocks vWf binding to GPIIb/IIIa,[6] interfered, however, with the appearance of vWf on the surface of ADP-stimulated platelets. We also demonstrated that inhibition of platelet secretion by incubation of PRP with aspirin and PGE_1 prior to stimulation inhibited ADP-induced binding of vWf.

Taken together, these studies suggest that vWf may be transferred to the platelet surface already bound to a receptor site during ADP activation in plasma. Because it has already been shown that α-granule membrane proteins can be found on the platelet surface following activation,[20] we suspect that this binding site could be the α-granule GPIIb/IIIa. However, our studies did not allow us to determine this specifically. Platelets may also have internal stores of GPIb;[21] but, GPIb has not been shown to be associated with α-granule membranes, and therefore we believe it less likely to be the site of vWf binding following ADP stimulation. vWf binding to an α-granule–derived receptor prior to surface association may explain why Parker et al were unable to achieve total blocking of binding of platelet vWf to thrombin-stimulated platelets activated in buffer containing the anti-GPIIb/IIIa antibody 10E5.[4]

An alternative mechanism for binding of secreted vWf could also exist. α-Granules contain a unique family of very high-mol-wt vWf multimers not present in plasma that have enhanced platelet binding characteristics.[18] These multimers, released into the plasma following platelet activation, may be able to compete effectively with fibrinogen for platelet GPIIb/IIIa binding sites. Our experiments with anti-GPIIb/IIIa and anti-GPIb antibodies suggest that this does not occur on the platelet surface; however, binding may occur within the platelet canalicular system and the blocking antibodies may not gain access to this space.

Our data corroborate those of other researchers indicating that vWf can bind to stimulated platelets and demonstrate that vWf is present in increased amount on the surface of platelets stimulated by ADP in plasma. The findings are consistent with the observation that vWf binds to ADP-stimulated platelets in PRP prepared from individuals with congenital afibrinogenemia.[7,22] In these individuals, vWf binding to GPIIb/IIIa partially substitutes for fibrinogen in supporting platelet aggregate formation. Whether platelet surface association of secreted vWf contributes to platelet aggregate formation or serves to enhance platelet adhesion in normal individuals remains to be determined.

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Cytogenetic and Cytochemical Studies on Progenitor Cells of Primary Acquired Sideroblastic Anemia (PASA): Involvement of Multipotent Myeloid Stem Cells in PASA Clone and Mosaicism With Normal Clone

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By cytogenetic and cytochemical analyses of individual hematopoietic colonies, we investigated clonality in progenitor compartments of primary acquired sideroblastic anemia (PASA). Two of our four subjects had reduced but countable numbers of CFU-E, BFU-E, and GFU-GM in methylcellulose culture. In one patient with cytogenetic abnormality of 47, XX, +8 in 67% of the bone marrow cells, cytogenetic analysis of individual erythroid bursts and granulocyte/macrophage colonies demonstrated two populations with and without 8 trisomy, the trisomy clone being 38% in BFU-E and 50% in CFU-GM. These findings indicate involvement of multipotent stem cells in PASA clone and mosaicism of two distinct populations in erythroid as well as granulocyte/macrophage progenitor compartments, the abnormal PASA clone and probably the normal clones. In another case with no cytogenetic abnormality, repeated iron staining showed that 31% to 40% of CFU-E and 25% to 54% of BFU-E had erythroblasts with heavy iron deposits. An ultrastructural analysis of 25 individual erythroid bursts revealed that 32% had highly dysplastic erythroblasts with marked ferruginous iron accumulation in the mitochondria. The other 68% and 15 normal bursts from a healthy control did not have noticeable dysplastic changes and iron deposits in the mitochondria. This cytochemical/ultrastructural mosaicism seems to be compatible with the cytogenetic mosaicism. However, whether the BFU-E derived from abnormal PASA clone selectively manifest iron accumulation in the mitochondria or whether the PASA clone itself shows variable degrees of abnormal iron metabolism remains to be determined by simultaneous performance of ultrastructural and cytogenetic analysis for single bursts.

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PRIMARY ACQUIRED sideroblastic anemia (PASA) is defined as refractory anemia with cellular bone marrow with erythroid hyperplasia and increased number of ringed sideroblasts. These cells have been considered pathognomonic of this disease since the early descriptions by Bjorkman,[1] Dacie et al[2] and Heilmeyer.[3] Ultrastructural studies established that an accumulation of iron in the mitochondria gives rise to the ring-form deposits,[4,5] and in some cases enzymatic analysis disclosed abnormalities of the heme synthetic pathway inside the mitochondria, such as reduced δ-aminolevurinic acid synthetase (ALA-S) activity.[6,7] It has long been known that some PASA patients later develop acute leukemia,[2,8-10] indicating the preleukemic nature of PASA. Cytogenetic studies have revealed that the abnormal hematopoiesis of PASA is a clonal disorder, as in other myelodysplastic syndromes.[11-15] Prchal et al recently analyzed G6PD isozymes of each cell lineage of mature blood cells, including lymphocytes from a female patient heterozygous for the G6PD locus, and showed that the PASA clone originates in a pluripotent stem cell.[16]

The blood picture of PASA is usually characterized by dimorphism of RBC morphology, which means mosaicism of hypochromic and normochromic cells.[2,10,17,18] It is also easy to find mosaicism of erythroblasts with and without ring-form iron deposits in the bone marrow. The pathophysiologic significance of these findings is not yet known. To investigate clonality in progenitor compartments, we performed cytogenetic analysis of individual hematopoietic colonies obtained from a patient with abnormal karyotype in the bone marrow cells. We also analyzed iron accumulation in single erythroid colonies obtained from another patient, using cytochemical and ultrastructural methods. We found evidence for involvement of multipotent stem cells in PASA clone and for clonal mosaicism with normal progenitor cells.

MATERIALS AND METHODS

Patients

Three patients (cases 1, 2, and 3), whose hematologic findings fulfilled the French-American-British (FAB) criteria for PASA (refractory anemia with ringed sideroblasts),[19] and one patient (case 4) with less prominent ringed sideroblasts were studied. The hematologic data for these patients at the time of the first colony assay are summarized in Table 1. None of these patients had a family history of sideroblastic anemia or underlying hematologic or nonhematologic disease. Cases 1, 2, and 3 showed a slight to moderate degrees of morphological dysplastic changes[19] in erythroid and granulocytic lineages. Case 4 manifested severe dysplastic changes in all cell lineages, including megakaryocytes. All cases had high serum iron levels (256 to 298 μg/dL) and high transferrin saturation (92% to 95%). Bone marrow samples were obtained from the sternum from each patient and one healthy control with informed consent.

Colony Assay

Erythroid colony assay (BFU-E and CFU-E). The method of Iscove et al was used.[20] 2 × 10^5 bone marrow buffy coat cells were

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Table 1. Data of Hematologic Examination and Colony Assay

Factor	Case 1	Case 2	Case 3	Case 4
Age and sex	64 F	76 F	30 F	74 F
History of anemia (yr)	7	12	7	5
Hb (g/dL)	9.4	9.0	6.9	6.5
WBC ($\times 10^9$/L)	2.9	3.05	1.9	5.6
Stab + segment (%)	48.0	48.0	36.0	65.0
Platelet ($\times 10^9$/L)	250.0	170.0	80.0	186.0
Ratio M:E	0.5	1.2	0.8	3.2
Ringed sideroblast (%)*	30	63	67	32
Chromosome abnormality	Yes	No	No	No
Transfusion requirement	Frequent	No	Frequent	Frequent
CFU-GM	20 ± 2	30 ± 4	6 ± 2	26 ± 1
CFU-E	24 ± 4	12 ± 4	6 ± 1	0
BFU-E	9.5 ± 0.5	40 ± 1	0	0

Abbreviations: Hb, hemoglobin; M:E, myeloid:erythroid.

Each value of CFU shows the mean of triplicate cultures, Normal value for CFU-GM 117 to 210 (mean 160)/1 × 10^5 light-density bone marrow cells, CFU-E 145-230 (mean 182)/2 × 10^5 bone marrow buffy coat cells, and BFU-E 72 to 122 (mean 94)/2 × 10^5 bone marrow buffy coat cells.

*Percentages among 100 erythroblasts.

plated in a 35-mm Falcon plastic dish containing 1.0 mL of α-medium with final concentrations of 0.88% methylcellulose, 30% fetal calf serum (FCS), 1% deionized bovine serum albumin (BSA) (GIBCO, Grand Island, NY), and 1.0 U/mL erythropoietin (Ep, Step III, Connaught Labs Toronto, Canada). For testing the response to various doses of Ep by BFU-E from the bone marrow in case 2, EP was added to give final concentrations of 0, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 U/mL. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. CFU-E were counted on day 7, and BFU-E were counted on day 14.

Granulocyte/macrophage colony assay (CFU-GM). The method of Iscove et al was used with some modification.[21] In brief, 1 × 10^5 light density (1.077/cm^3) mononuclear cells separated from bone marrow specimen, were plated in a 35-mm Falcon plastic dish containing 1.0 mL of α-medium with 0.88% methylcellulose, 20% FCS, and 10% giant-tumor cell-conditioned medium (GIBCO). After 8 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, cell aggregates with >20 cells were counted as CFU-GM.

Cytogenetic Investigation

Bone marrow cells were directly processed for chromosomal preparation, and cytogenetic analyses were peformed using the standard method and/or the G-banding method.[22]

Chromosome analysis from individual colonies was performed using our own procedure described elsewhere.[23] In brief, individual colonies were aspirated by a finely drawn-out capillary tube, and transferred into a droplet (10 μL) of 0.075 mol/L KCl solution placed on a poly-L-Lysine (Sigma Chemical, St Louis) coated slide. After thorough mixing, the slide was turned upside down to let the droplet hang downward and allowed to stand at 37°C in a wet-chamber for 20 minutes to obtain maximal hypotonic effects. The slide was then turned again, the droplet being upward, and allowed to stand for 15 minutes to let the cells attach to the slide. The fixation process consisted of three steps: First, a small amount (10 μL) of 30% fixative (3:1 methanol:acetic acid) diluted with 0.075 mol/L KCl was added (5 minutes); second, three droplets of 20% ethanol diluted with 0.075 mol/L KCl were added using a Pasteur pipette and allowed to stand for 10 minutes; third, the entire slide was immersed in fresh 100% fixative and allowed to stand for >10 minutes. The slide was flame-dried and stained with Giemsa and/or Q-banded.

Cytochemical Investigation

Perls' reaction for iron[24] was used on air-dried smears of bone marrow cells and Cytospin (Shandon Southern Products, Cheshire, England) smears of single CFU-E and BFU-E. Cytospin-smears were made by aspirating each colony or burst using a finely-drawn-out capillary tube and mixing it with 10 μL of α-medium on a slide glass. The slide was cytocentrifuged at 1,200 rpm for three minutes. Blue granules or deposits were considered accumulated iron in the cytoplasm. Erythroblasts with iron granules arranged along more than one-third of the nuclear outline were defined as ring-form.

Ultrastructural Investigation

For TEM analysis of single BFU-E in case 2 and of one healthy control, 0.5 mL 2.0% glutaraldehyde in 0.1 mol/L phosphate buffer was gently added to dishes for erythroid colonies on day 14. Each erythroid burst became dark and hard after 20 minutes. This made easier aspiration of single bursts using finely-drawn-out capillary tubes. Each burst was transferred into 0.5 mL of 0.1 M phosphate buffer in a multiwell glass plate. Three changes of the buffer were made, and 0.2 mL of 2% OsO_4 was added. After 15 minutes, three changes of the buffer were repeated. Single bursts were then dehydrated in six grades of ethanol from 30% to 100% (10 minutes each). Embedding of each burst in Epon was done by standard method[25] under a dissecting microscope. Ultrathin sections were made on a LKB 4800 (LKB, Bromma, Sweden). The double-counterstain with uranyl acetate and lead citrate was used, and TEM observation followed on a JEM-100B(JEOL, Tokyo). To confirm that high-electron-dense deposits in the mitochondria of the erythroblasts were actually iron accumulation, X-ray microprobe analysis was done using a JEM-100CX (JEOL).

RESULTS

BFU-E, CFU-E, and CFU-GM Enumeration

Numbers of CFU-E and BFU-E were relatively high (though far below normal) in cases 1 and 2 (Table 1). A few degenerative CFU-E and no BFU-E were formed in case 3. No cellular aggregations were present in case 4. Spontaneous erythroid colony formation was absent in all cases when Ep was not added. CFU-GM formed fairly well in all cases, but in reduced numbers.

Cytogenetic Analysis of Bone Marrow Cells and Individual Hematopoietic Colonies in Case 1

The bone marrow direct method revealed that nine (33.0%) of 27 metaphases were aneuploidy of 47, XX, +8, and 18 (67.0%) normal karyotypes. Cytogenetic analysis of individual erythroid bursts and granulocyte/macrophage colonies demonstrated the same mosaic condition in both BFU-E and CFU-GM compartments (Table 2). In a few individual bursts and GM-colonies, two or more metaphases could be analyzed. They all showed either normal or abnormal karyotype.

Table 2. Cytogenetic Analysis of Individual Colonies in Case 1

Colony Type	No. of Colonies (Metaphase) Examined	Colony With 46,XX		Colony With 47,XX, +8	
		n (%)	No. of Normal Metaphases/ No. of Metaphases Examined	n (%)	No. of Abnormal Metaphases/ No. of Metaphases Examined
BFU-E	8 (22)	5 (62.0)	3/3		
			2/2	3 (38.0)	5/5
			2/2		4/4
			2/2		1/1
			2/2		
CFU-GM	10 (18)	5 (50.0)	2/2		4/4*
			2/2		3/3
			2/2	5 (50.0)	1/1
			1/1		1/1
			1/1		1/1

*This colony showed 48,XX, +8, +F, ie, an additional change.

Table 3. Iron Deposit Analysis of Individual Erythroid Colonies (Case 2)

	Light-Microscopic Analysis*		Ultrastructural Analysis
	No. of Fe$^+$ Colonies/ No. of Colonies analyzed (%)		No. of Mitochondrial Fe$^+$ colonies/ No. of Colonies Analyzed (%)
Date	CFU-E	BFU-E	BFU-E
April 15, 1983	—	24/78 (30.8%)	8/25 (32%)
March 5, 1984	6/15 (40%)	16/30 (54.2%)	—
September 8	9/29 (31%)	15/63 (23.8%)	—
Normal control	0/15 (0%)	0/15 (0%)	1†/15 (6.6%)

*Only colonies with coarse iron granules (Fe$^+$) in the cytoplasm of colony-composing erythroblasts were regarded as abnormal.

†Only one normal BFU-E showed ferruginous iron accumulation in a few mitochondria of two erythroblasts among ~300 burst-composing cells.

Cytochemical Analysis of Single Erythroid Colonies and Bursts in Case 2

The results of serial cytochemical investigations on the iron deposits in individual erythroid colonies and bursts from case 2 are shown in Table 3. In the first analysis, ~30% of bursts were iron deposit positive. In some bursts, all the erythroblasts had heavy deposits of iron, but in others only a part (30% to 60%) of the erythroblasts had detectable amounts of such deposits. In some erythroblasts, iron granules were in ring-form like the ringed sideroblasts of the bone marrow smears, but in others they were scattered in the cytoplasm, making it difficult to consider them equivalent to ringed sideroblasts (Fig 1). Ultrastructural study was therefore needed to find the actual site of iron accumulation. In the second and third analyses, iron-positive and iron-negative populations were observed in both CFU-E and BFU-E compartments.

Ultrastructural Analysis of Single Erythroid Bursts in Case 2

The bursts could be classified into two distinct populations, one with ferruginous iron deposits inside the mitochondria (Fig 2A and B), which we called Fe-positive, and the other completely without iron deposits in the mitochondria (Fig 3), which we called Fe-negative. In the Fe-positive population, there were few iron deposits outside the mitochondria; only occasional ferritin clusters were seen (Fig 2B), suggesting that the scattered pattern of iron deposits observed in the cytochemical preparations of single colonies or bursts indicates that most of the iron has actually accumulated in the mitochondria. The percentage of Fe-positive bursts identified cytochemically, including both ring-forms and scattered-forms, was close to that identified by TEM (Table 3). X-ray microprobe analysis confirmed that the electron-dense deposits in the mitochondria were indeed ferruginous.

In each Fe-positive burst, almost all erythroblasts had slight (containing thin iron deposit layer along cristae) to extensive (containing heavy iron deposit filling the space between cristae) iron accumulation in almost all visible

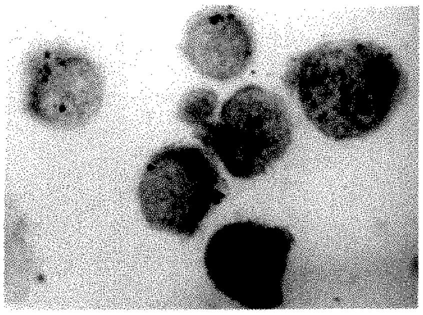

Fig 1. Cytospin-smear of an erythroid burst from case 2 stained for iron (Perls' reaction). The coarse, blue iron granules are scattered in the cytoplasm. Only a few erythroblasts in this burst show ring-form iron deposits. (Original magnification ×1,000; current magnification ×500.)

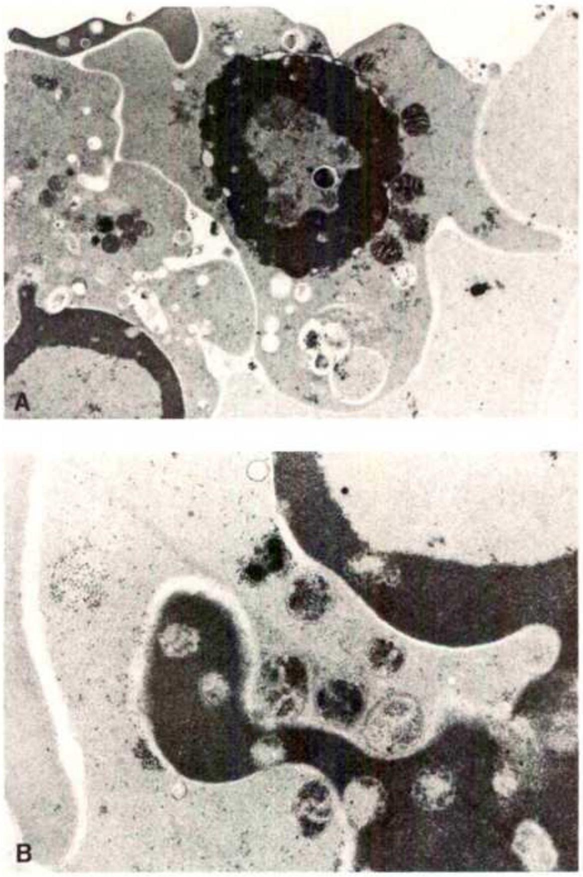

Fig 2. TEM of a day-14 erythroid burst from case 2, with heavy iron deposits. (A) The erythroblasts show marked dysplastic changes, as well as typical ring-form iron deposits. There is marked disintegration of the chromatin structure, disappearance of the nuclear envelope, scanty or almost no ribosomes, vacuoles in the cytoplasm and in the nucleus, and severe degeneration of the mitochondria (vacuolation and disruption). (Original magnification ×5,000; current magnification ×2,500). (B) In some erythroblasts, a few ferritin clusters are seen outside the mitochondria. The ribosomes are visible but few, with no polyribosome formation, suggesting that there are multiple background disorders in the cellular biochemistry of PASA erythroblasts (Original magnification ×13,000; current magnification ×6,500).

mitochondria, indicating that TEM analysis was more sensitive than light-microscopic cytochemistry for the detection of iron deposits in the cytoplasm. At the same time, severe dysplastic changes were observed. In contrast, in bursts with only Fe-negative erythroblasts, the cells had almost normal ultrastructural appearance without any dysplastic changes. Fourteen of 15 bursts from a normal control had Fe-negative erythroblasts, and all cells seemed ultrastructurally normal (Fig 4A). In one burst, however, a few erythroblasts had thin iron deposits in mitochondria (Fig 4B).

Erythropoietin Sensitivity of Erythroid Progenitors in Case 2

As shown in Table 4, the numbers of total CFU-E and Fe-positive CFU-E increased proportionately to increasing doses of Ep and reached a plateau at 1.0 U/mL. Thus, there

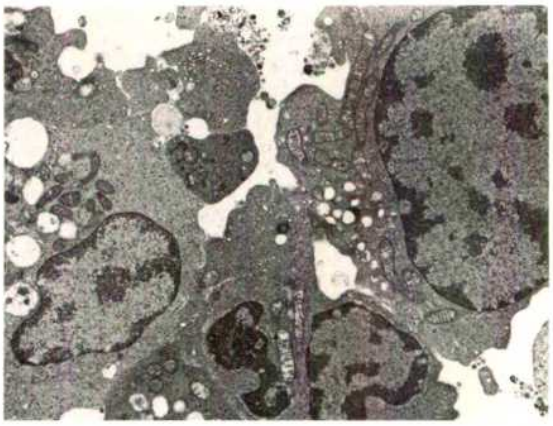

Fig 3. TEM of a day-14 burst from case 2, with normal appearance. The erythroblasts show almost normal ultrastructural morphology; there are abundant polyribosomes and mitochondria with no iron accumulation (Original magnification ×5,500; current magnification ×2,750).

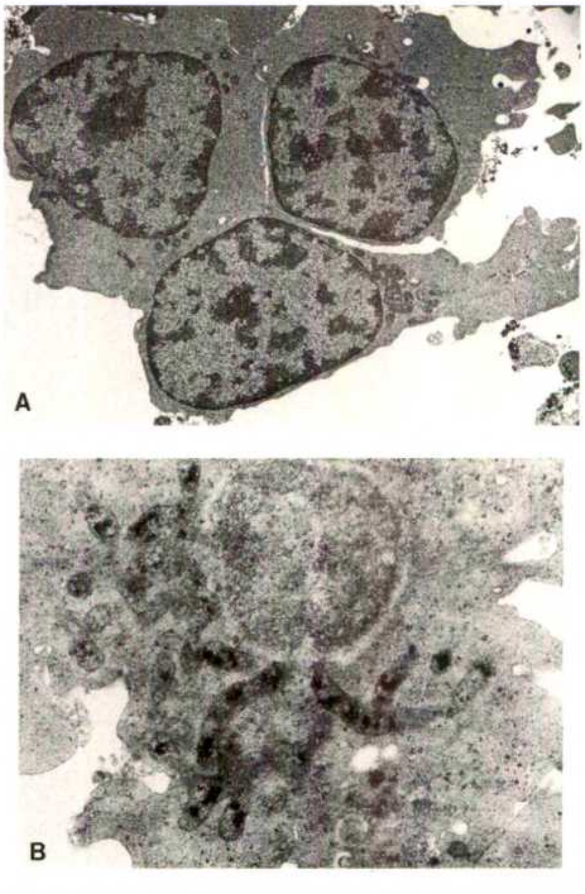

Fig 4. TEM of a day-14 burst from a normal control. (A) The erythroblasts show entirely normal appearance with no karyorrhexis and no abnormal iron deposits in the cytoplasm or in the mitochondria. (B) In one burst in 15, a few erythroblasts among some 300 cells observed had iron deposits in the mitochondria.

Table 4. Erythropoietin-Dose Response of Erythroid Progenitors (Case 2)

Erythropoietin Dose (U/mL)	CFU-E No. 2 × 10^5 Cells Total	CFU-E No. 2 × 10^5 Cells Fe$^+$ (%)	BFU-E No. 2 × 10^5 Cells Total	BFU-E No. 2 × 10^5 Cells Fe$^+$ (%)	
0.1	12.6 ± 0.6	3.3 ± 1.5 (26.2)	3.7 ± 1.2	3.0 ± 1.0 (81.0)	
0.5	32.7 ± 1.5	6.0 ± 1.7 (18.3)	16.0 ± 1.7	5.7 ± 0.5 (35.6)	1
1.0	36.3 ± 1.5	12.3 ± 1.2 (33.9)	17.3 ± 1.5	10.3 ± 1.5 (59.5)	2
1.5	34.3 ± 1.5	11.7 ± 2.0 (34.1)	20.6 ± 1.5	11.3 ± 1.5 (54.8)	3
2.0	34.7 ± 1.4	12.3 ± 2.5 (35.4)	32.3 ± 1.5	18.3 ± 0.5 (56.6)	4

Numbers of CFU-E and BFU-E are the mean ± 1 SD of triplicate culture. All CFU-E and BFU-E enumerated were aspirated individually and stained for iron. The percentage of Fe$^+$ BFU-E at erythropoietin 0.1 U/mL was compared with those at higher doses using Fisher's exact test. (1, $P = .0065$; 2, $P = .1478$, 3, $P = .0867$; 4, $P = .0970$).

was no difference in Ep sensitivity between Fe-positive and Fe-negative CFU-E compartments. In contrast, Fe-positive BFU-E seemed to be more sensitive to Ep since Fe-positive BFU-E grew predominantly at 0.1 U/mL. The difference in percentage of Fe-positive BFU-E was statistically significant, however, only between 0.1 and 0.5 U/mL.

DISCUSSION

The present cytogenetic study on individual hematopoietic colonies revealed in case 1 the presence of an identical aneuploid clone in two different cell lineages, providing evidence that the abnormal clone of PASA involves multipotent hematopoietic stem cells. This cytogenetic finding is compatible with the observation by Prchal et al using G6PD isozyme analysis.[16] Their conclusion was that all blood formed elements, including lymphocytes, are derived from the abnormal PASA clone that originated in a pluripotent stem cell. Our study further revealed karyotypic mosaicism in erythroid as well as granulocyte/macrophage progenitor cell compartments. Mosaicism was also present in the direct bone marrow sample. Many cytogenetic studies on PASA bone marrows also disclosed mosaicism.[11-15] Because Prchal et al did not analyze hematopoietic colonies enzymatically, it is not clear whether there was a heterogeneous composition in progenitor compartments in their case. The discrepancy between our results and Prchal's may in part reflect the different stages of PASA at which clonal study was performed. In our series, cases 1 and 2 showed a progressive declining of erythroid burst formation (data not shown), suggesting expansion of the PASA clone.

We obtained higher percentages of population with normal karyotype in erythroid and GM progenitor compartments than in the direct bone marrow sample. It appears possible in vitro to obtain colonies of which progenitor cells belong to normal clones but are inhibited in vivo and unable to deliver normal mature progeny. A similar situation was recently observed in some patients with chronic myeloid leukemia by Dube et al.[26] Our observation in case 2 that Fe-positive BFU-E seemed to have hypersensitivity to Ep may provide, if confirmed in other cases of PASA, a clue to elucidating the underlying mechanism of growth advantage by the PASA clone over normal clone. However, this does not necessarily explain the growth advantage in the multipotent stem cell compartment.

Demonstration of the normal clones remaining in PASA bone marrow as a minor population is important from a therapeutic viewpoint. Theoretically, chemotherapeutic or differentiation-induction regimens such as continuous low-dose cytosine arabinoside (LDAC) regimen may reverse the bone marrow with dominance of PASA clone to remission state. With the LDAC regimen, hematologic remission has been reported to be induced in some patients with PASA.[27,28] However, clonal alternation was not confirmed in these cases.

By combining clonal culture with cytochemical/ultrastructural methods, the present study disclosed in case 2 that Fe-positive and Fe-negative populations are present in the erythroid progenitor compartment of PASA bone marrow. Differences in ultrastructural morphology of erythroblasts in individual bursts were distinct between the two populations. Marked dysplastic changes were observed only in the erythroblasts of Fe-positive bursts, suggesting premature degradation of cells in vitro, which may lead to reduced number of erythroid bursts. This cytochemical/ultrastructural mosaicism in case 2 seems to be compatible with the cytogenetic mosaicism in case 1, Fe-positive bursts being derived from the PASA clone and Fe-negative bursts being derived from the normal clone. If this assumption is correct, one can explain the dimorphism of RBCs in PASA as expression of the clonal mosaicism in this disease. Because a few Fe-positive erythroblasts were observed in one BFU-E from normal control, there still remains a possibility that the mitochondrial iron deposit is not a clonal marker and that the PASA clone itself shows much diversity in degree of defective heme synthesis and produces maturing erythroblasts and RBCs with variable degrees of iron deposits and hemoglobin concentration, respectively.

Several reports describe the relationship between PASA clone and abnormal heme synthesis. Using biochemical methods, Ibrahim et al compared the ALA-S activity and C^{14}-ALA incorporation into heme of erythroblasts directly sampled from PASA bone marrow and of erythroid colonies obtained by culturing the same bone marrow; they found reduced ALA-S activity and C^{14} incorporation in the former but almost normal values in the latter[29,30] and postulated that these erythroid progenitors, biochemically normal in appearance, were derived from residual normal stem cells. Hutcheson et al did an ultrastructural study on erythroid bursts from PASA patients and demonstrated for the first time in vitro distinct iron accumulation in the mitochondria;[31] however, they processed groups of bursts for TEM study instead of analyzing them individually and did not observe bursts of normal appearance.

Mizoguchi et al undertook a light-microscopic/cytochemical investigation of CFU-E and BFU-E from PASA patients,[32] as we did. In one case, they found that all BFU-E were Fe-positive but contained two types of erythroblasts, with and without iron deposits, whereas all CFU-E were Fe-negative. They postulated that BFU-E of the PASA clone can give rise to both types of progenies and Fe-negative CFU-E are those PASA progenies with higher proliferative potential in vitro. Differences in technical sensitivity to iron detection (light-microscopic *v* ultrastructural methods) may account in part for the discrepancy between their work and ours. To establish the significance of ringed sideroblasts in the pathogenesis of PASA, one must perform cytogenetic or G6PD-isoenzyme anlaysis and ultrastructural observation simultaneously for single erythroid bursts.

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Phorbol Esters Sensitize Platelets to Activation by Physiological Agonists

By Wolfgang Siess and Eduardo G. Lapetina

Phorbol esters such as phorbol 12, 13-dibutyrate (PdBu; 40 to 200 nmol/L) or 12-*O*-tetradecanoyl phorbol 13-acetate (20 to 80 nmol/L) added to aspirinized platelet-rich plasma (PRP) 5 to 15 seconds prior to various platelet stimuli (epinephrine, ADP, prostaglandin endoperoxide analog U44069, collagen, PAF, or vasopressin) potentiate the rate and extent of aggregation and ATP secretion induced by those agonists. Platelet aggregation, but not secretion, is potentiated at low concentrations of agonists; platelet secretion is potentiated at higher concentrations of the platelet stimuli. Potentiation of platelet responses was also observed when the preincubation time with PdBu was extended to 12 minutes and also occurred in washed platelets. The potentiating effect of phorbol esters is not mediated by formation of arachidonate metabolites or by released ADP. The sensitizing effect of PdBu on platelet aggregation induced by epinephrine is unique, since in contrast to the other platelet stimuli it is also found at maximal concentrations of epinephrine and does not diminish with prolonged preincubation of platelets with PdBu. Activation of protein kinase C ranges from 20% to 80% over control after 1 to 10 minutes of platelet pretreatment with PdBu but dramatically increases after subsequent addition of a stimulus such as vasopressin. In contrast, agonist-induced myosin light chain phosphorylation is reduced after platelet pretreatment with PdBu. The results indicate that protein kinase C activation enhances platelet aggregation and dense granule secretion triggered by physiologic stimuli, although it desensitizes agonist-induced myosin light chain phosphorylation.
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TWO FUNCTIONS of protein kinase C for platelet activation have been suggested: (a) synergism with Ca^{2+} to induce platelet aggregation and secretion,[1,2] and (b) desensitization of platelet responses to receptor-activating platelet stimuli.[3-12]

Bioactive diacylglycerols or phorbol esters induce either no platelet response or only a small, delayed aggregation and secretion response at concentrations that fully activate protein kinase C. They will, however, evoke rapid, full secretion and aggregation if added together with subthreshold concentrations of Ca^{2+} ionophores.[1,2] Preincubation of platelets with phorbol ester or bioactive diacylglycerols for 1 to 9 minutes causes an inhibition of phospholipase C-induced inositol phospholipid hydrolysis, Ca^{2+} mobilization, thromboxane B_2 (TXB_2) formation, shape change, granule secretion and aggregation induced by various physiological stimuli such as thrombin, vasopressin, PAF or ADP.[4-12] Although 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) added up to 1 minute before thrombin accelerates thrombin-induced dense granule secretion, phosphatidic acid formation, and breakdown of phosphatidylinositol 4,5-bisphosphate of washed platelets incubated at 23°C,[5] most studies have reported and stressed the desensitizing effect of protein kinase C activation on agonist-induced platelet responses. Recent studies have not clarified the situation. On one hand, by incubating platelets for only a short time with TPA (10 seconds before the stimulus), a clear inhibition of thrombin- or ADP-induced shape change, Ca^{2+} mobilization and myosin association with the cytoskeleton was found.[9] Preincubation of platelets with TPA for 1 to 5 minutes before the agonist has also been reported to inhibit shape change, aggregation and ATP-secretion in response to thrombin, PAF, ADP, or U46619 in the presence of extracellular Ca^{2+},[10,11] but it induces a potentiation of ATP secretion in the absence of extracellular Ca^{2+} (<100 nmol/L).[10] On the other hand, other investigators found only a small reduction of agonist-stimulated serotonin release and no inhibition of cytosolic Ca^{2+} increase, myosin light chain phosphorylation, or cytoskeletal assembly after preincubating platelets with TPA for 2 minutes in the presence of extracellular Ca^{2+} (0.05 mmol/L).[12] When TPA was added 20 seconds before a secondary challenge with arachidonic acid or thrombin, an enhanced rate of secretion was observed.[12] Another group found that preincubation (10 seconds to 2 minutes) of platelets with TPA caused a potentiation of serotonin secretion induced by thrombin or U46619 but an inhibition of arachidonate release, TXB_2-formation, Ca^{2+} mobilization, and α-granule secretion.[13] The situation was further complicated if 1-oleoyl,2-acetylglycerol (OAG) was used, since the effects observed were different from those caused by phorbol ester: If added 10 seconds to 5 minutes before or 10 to 60 seconds after thrombin, OAG potentiated thrombin-induced aggregation and serotonin secretion without affecting the Ca^{2+} mobilization.[14]

A clear picture of the role of protein kinase C activation for platelet responses triggered by physiologic agonists has not emerged from these studies. Because all studies (except part of one study, ref. 13) were done using washed platelets, and the washing procedure and type of resuspension buffer may have introduced problems of nonphysiologic platelet behavior, we decided (a) to investigate the effect of phorbol ester on agonist-induced platelet activation in PRP, and (b) to find a method of preparing washed platelets that gave results similar to those obtained in PRP.

MATERIALS AND METHODS

Materials. 4α-Phorbol, 4α-phorbol 12, 13-didecanoate (4α-PDD), phorbol 12, 13-dibutyrate (PdBu), TPA [identical to phorbol 12-myristate, 13-acetate, (PMA)], and prostaglandin E_1 were pur-

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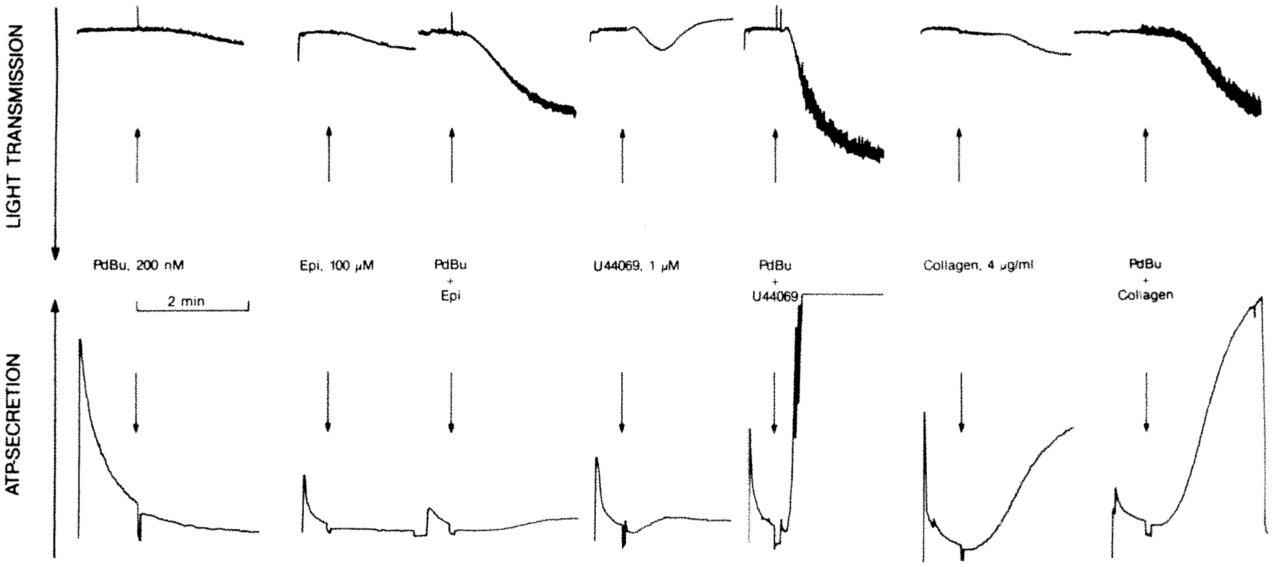

Fig 1. Tracings of aggregation and ATP secretion of aspirinized PRP stimulated by various agonists in the presence or absence of PdBu. PdBu was added simultaneously with the agonists.

chased from Sigma (St Louis). All other substances were obtained as previously described.[15-17]

PRP. PRP was prepared by centrifugation (200 g for 20 minutes) from 120 mL freshly drawn human blood anticoagulated with $^1/_{10}$ vol trisodium citrate (3.8%). PRP was incubated with 1 mmol/L acetylsalicylic acid (aspirin; dissolved as 1 mol/L solution in ethanol) for 15 minutes at 37°C in most experiments and then capped and stored at room temperature. To exclude direct influence of aspirin in plasma on the results obtained, in some experiments (results shown in Figs 1-9) platelets were pelleted by centrifugation (800 g for 10 minutes) after addition of PGI_2 (20 ng/mL) and resuspended in autologous platelet-poor plasma (PPP) containing creatine phosphate (CP) (1 mmol/L) and creatine phosphokinase (CPK) (10 U/mL).

Washed platelets. In studies using washed platelets, PRP—after aspirinization and addition of PGI_2 (20 ng/mL)—was centrifuged, washed once with 10 mL buffer A (20 mmol/L HEPES, 138 mmol/L NaCl, 2.9 mmol/L KCl, 3.3 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, pH 7.4) containing apyrase (0.6 U/mL

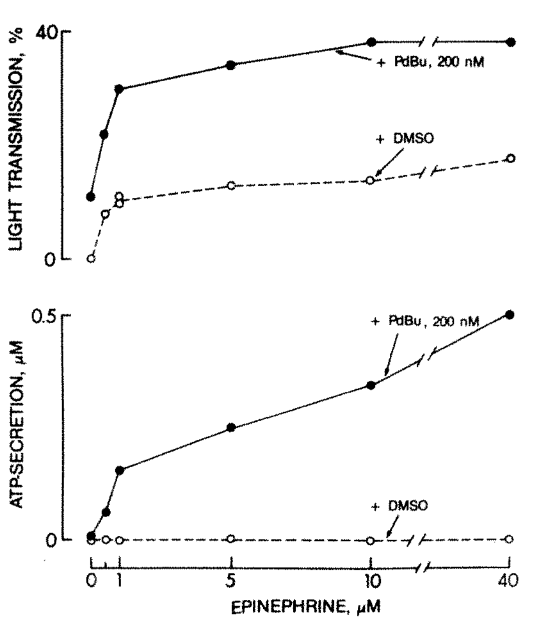

Fig 2. Potentiation of epinephrine-induced platelet responses by PdBu. PdBu (100 nmol/L) or DMSO was added 5 seconds before epinephrine to aspirinized PRP containing CP (1 mmol/L), CPK (10 U/mL). Aggregation and ATP secretion were measured 2 minutes after addition of the agonist. The samples containing DMSO gave results identical to samples without DMSO.

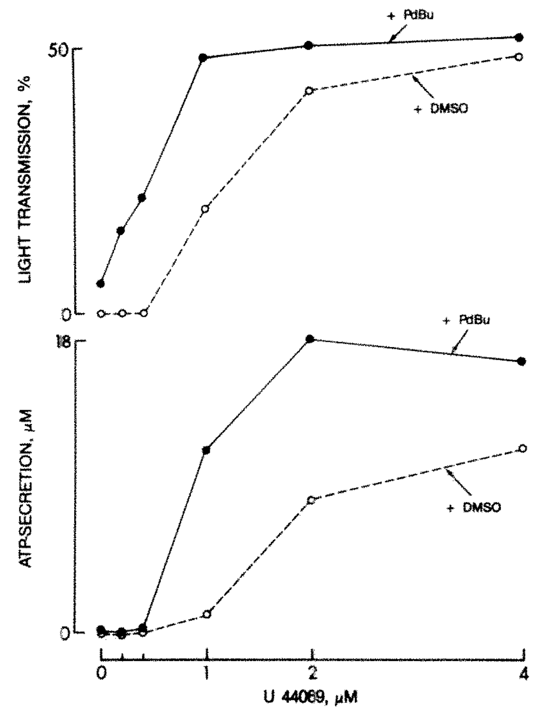

Fig 3. Potentiation of U44069-induced platelet responses by PdBu (200 nmol/L). Conditions were the same as those described in the legend to Fig 2.

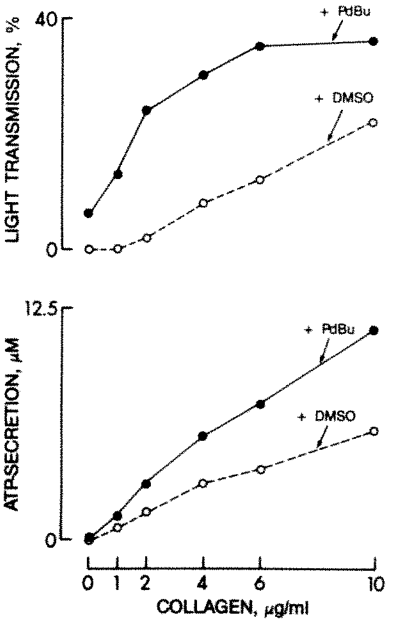

Fig 4. Potentiation of collagen-induced platelet responses by PdBu (200 nmol/L). Conditions were the same as those described in the legend to Fig 2.

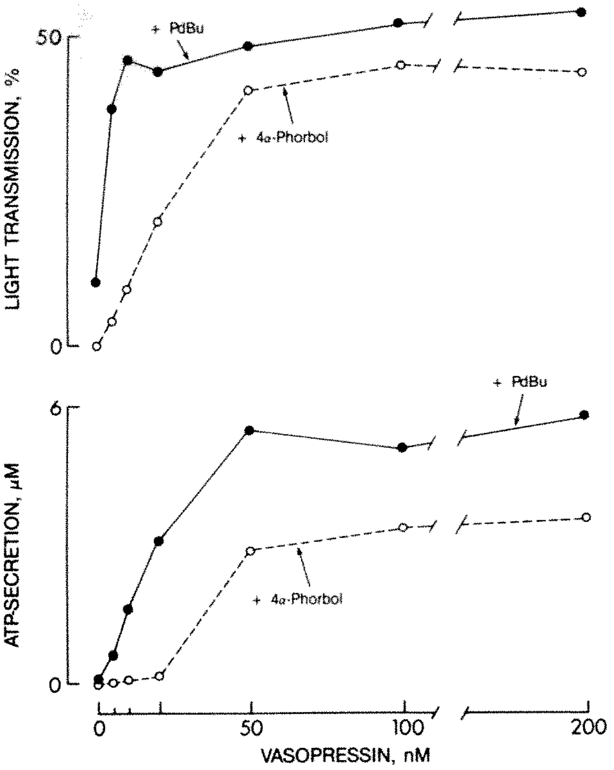

Fig 5. Potentiation of vasopressin-induced platelet responses by PdBu (200 nmol/L). Conditions were the same as those described in the legend to Fig 2. The samples containing 4α-phorbol gave results identical to controls.

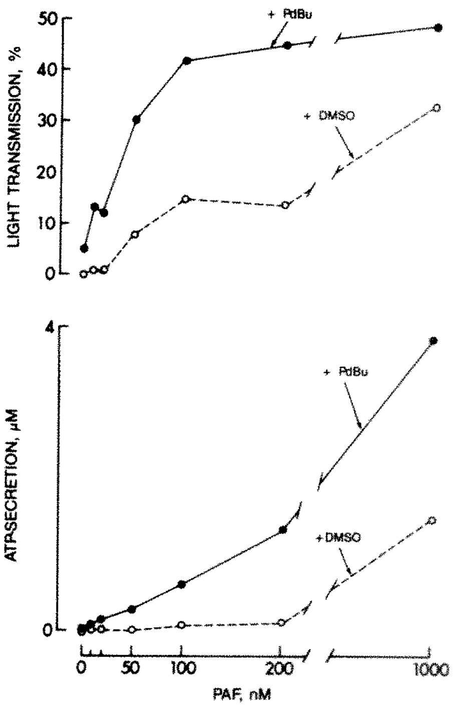

Fig 6. Potentiation of PAF-induced platelet responses by PdBu (200 nmol/L). Conditions were the same as those described in the legend to Fig 2.

ADP'ase) and PGI$_2$ (50 ng/mL), and finally resuspended in 20 mL of buffer B without apyrase, PGI$_2$ and EGTA, but with CP (1 mmol/L), CPK (10 U/mL) and CaCl$_2$ (0.1 mmol/L). PPP at a final concentration of 5% was added 1 minute before the stimulus.

Protein phosphorylation. PRP from 120 mL of blood—after aspirinization and addition of PGI$_2$ (20 ng/mL)—was centrifuged. Platelets were resuspended in 2 mL autologous PPP containing apyrase (3 U/mL ADP'ase) and 1 μmol/L PGE$_1$, and they were incubated with 8 to 10 mCi ^{32}P for 1.5 hours at 37°C. Then 20 mL of prewarmed (37°C) buffer A containing PGI$_2$ (50 ng/mL) was added, and platelets were pelleted by centrifugation (800 g for 10 minutes). Platelets were resuspended in 15 to 20 mL buffer without EGTA or PGI$_2$ but with apyrase (0.6 U/mL ADP'ase) and 5% PPP. The platelet suspension was kept at room temperature. Samples (0.6

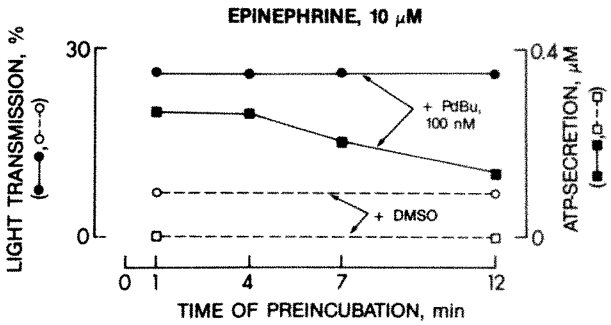

Fig 7. Effect of time of platelet pretreatment with PdBu on epinephrine-induced platelet responses in PRP. Aspirinized PRP containing CP, CPK was preincubated with PdBu at 37°C for the time periods indicated before the addition of epinephrine. Platelet responses were measured 2 minutes after addition of epinephrine.

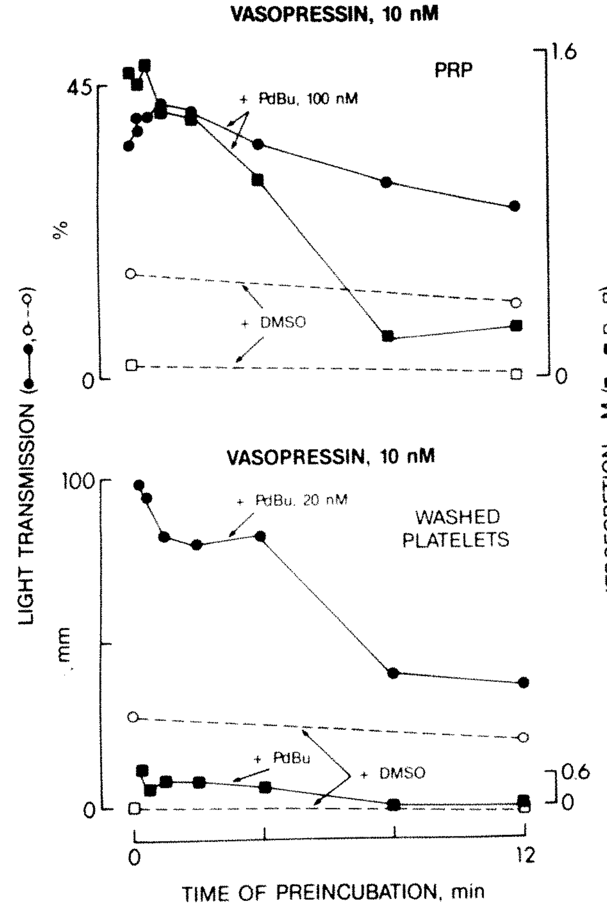

Fig 8. Comparison of platelet sensitization by PdBu on responses of PRP and washed platelets induced by vasopressin—effect of time length of platelet pretreatment with PdBu. Details are given in the Materials and Methods section.

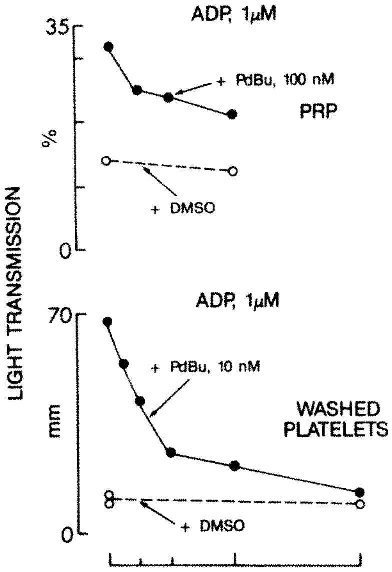

Fig 9. Effect of time of pretreatment with PdBu on responses of PRP and washed platelets induced by ADP.

mL) were transferred into aggregometer cuvettes and incubated with PdBu, 4-α-PDD or DMSO (1 μL) for 10 seconds to 10 minutes at 37°C before addition of the platelet stimulus. Aliquots (0.05 mL) were transferred into 0.05 mL of sample buffer containing 1% sodium dodecyl sulfate (SDS) and dithiothreitol (15 mg/mL) for measurement of protein phosphorylation. Phosphorylation of ^{32}P-labeled proteins was measured after separation on SDS-polyacrylamide (12.5%) gel electrophoresis (PAGE).[17]

Platelet aggregation and secretion. Platelet aggregation was measured using either a single-channel aggregometer adjusted to maximal sensitivity or a Lumi-Aggregometer (Chronolog, Havertown, PA). The Lumi-Aggregometer was calibrated in studies using PRP with autologous PPP (0.4 mL) and in studies using washed platelets with 0.3 mL platelet suspension diluted with 0.1 to 0.2 mL buffer. ATP secretion was measured in the Lumi-Aggregometer after addition of 0.04 mL of a mixture of Luciferin/Luciferase (Chronolog). PdBu or TPA (dissolved in DMSO) was added in small volumes (1 to 2 μL) using an Eppendorf microliter pipette.

Arachidonate metabolites. For measurement of production of arachidonate and arachidonate metabolites, PRP (50 mL) was centrifuged after addition of PGI_2 (50 ng/mL), resuspended in 5 mL PPP containing 1 μmol/L PGE_1 and apyrase (0.6 U ADP'ase/mL) and incubated with 100 μCi of [^{3}H]arachidonic acid (added in 0.02 mL ethanol) for 1.5 hours at 37°C. Platelets were centrifuged, washed twice with buffer A containing 10% PPP, apyrase, and PGI_2 (50 ng/mL) and resuspended in buffer B containing 10% PPP. $CaCl_2$ (0.1 mmol/L) was added 1 minute before the stimulus. Samples (0.5 mL) were stirred in the aggregometer at 37°C, and aliquots (0.1 mL) before (control) and 1 minute after addition of the stimulus were transferred into 0.6 mL of 3% glutaraldehyde/100 mmol/L $NaHPO_4$. Platelets were pelleted using a microfuge (12,000 g for 2 minutes), and the supernatant was counted for ^{3}H-radioactivity.

Data analysis. Data are shown for single experiments typical of at least three similar experiments. The extent of aggregation is either expressed as deflection of the recording pen in millimeters (when the more sensitive aggregometer was used) or in percent change of light transmission (when the Lumi-aggregometer was used). The rate of aggregation and secretion was estimated measuring the initial slope of the curves plotted against a time interval of 30 seconds.

RESULTS

Studies in PRP. PdBu (200 nmol/L) added to aspirinized platelets suspended in plasma containing CP/CPK induced a slow, limited aggregation response that started 45 to 60 seconds after addition. No ATP secretion was observed (Fig 1). If PdBu was added 5 to 15 seconds before or simultaneously with platelet stimuli such as epinephrine, endoperoxide analog U44069, collagen, vasopressin, PAF, or ADP, the rate and extent of both aggregation and ATP secretion induced by those agonists were greatly enhanced (Figs 1 through 6 and 10, Table 1). Also, PdBu (100 nmol/L) added to normal untreated citrated PRP, potentiated platelet aggregation induced by various agonists (Table 2). The potentiation of platelet aggregation and secretion by PdBu in response to epinephrine was unique, because it was observed at both low and high concentrations of epinephrine (Figs 2 and 11). Epinephrine alone induced only a small primary aggregation without ATP secretion (Fig 1). The PdBu-induced sensitization of platelets toward the other platelet stimuli was more pronounced when their concentrations were low (Figs 3 through 6). Time-course studies and

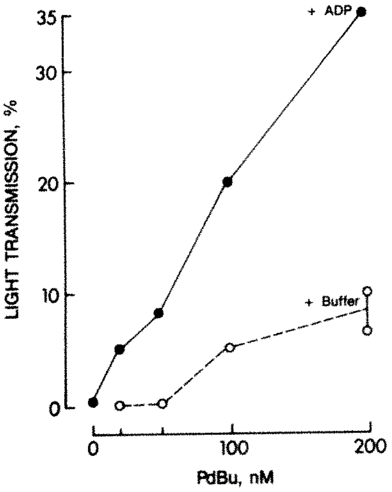

Fig 10. Potentiation of ADP-induced platelet aggregation by various concentrations of PdBu. ADP (0.5 μmol/L) or buffer was added 5 seconds after PdBu to PRP containing aspirin (1 mmol/L). Aggregation was measured 3 minutes after addition of ADP. ADP (0.5 μmol/L) alone induced shape change without aggregation.

Table 2. Potentiation of Stimulus-Induced Platelet Aggregation in PRP by PdBu

Stimulus	Aggregation (% Light Transmission) DMSO	4α-PDD	PdBu
Control	0	0	22 ± 1
Epinephrine 1 μmol/L	46 ± 3	48 ± 2	80 ± 4
ADP 0.5 μmol/L	44 ± 5*	43 ± 1*	83 ± 2
PAF 20 nmol/L	22 ± 4*	16 ± 2*	53 ± 2
Vasopressin 100 nmol/L	27 ± 2*	25 ± 2*	71 ± 3

Untreated citrated PRP (0.5 mL, 37 °C) was incubated for 10 seconds with either DMSO (1 μL), 100 nmol/L 4α-phorbol 12,13-didecanoate (4α-PDD) or 100 nmol/L PdBu before addition of various platelet stimuli. Aggregation was recorded for 1 minute using a Lumi-Aggregometer. For increase of sensitivity, the PPP cuvette contained 0.2 mL PPP plus 0.4 mL PRP. Results show triplicate values (mean ± SD) of one of three typical experiments.

*Reversible aggregation.

dose–response curves for the different platelet stimuli in the presence or absence of PdBu show that platelet aggregation was the first response potentiated by PdBu (Figs 1 and 3 through 6). Potentiation of ATP secretion by PdBu occurred at higher concentrations of the platelet stimulus and could even be observed at agonist concentrations that were maximal for aggregation (Figs 2 through 6). 4α-Phorbol and 4α-phorbol 12,13-didecanoate, inactive parent compounds of PdBu, were ineffective at inducing potentiation of platelet aggregation and ATP secretion (Figs 5 and 11 and Table 2).

Reversible aggregation responses induced by low concentrations of vasopressin, PAF, U44069, or ADP became irreversible in the presence of PdBu. The irreversibility of aggregation did not seem to depend on secretion, because irreversible aggregation induced by low concentrations of U44069 plus PdBu was not associated with ATP secretion, and reversible aggregation induced by PAF (1 μmol/L) was accompanied by ATP secretion (Figs 3 and 6).

The potentiating effect of phorbol ester on stimulus-induced aggregation was dependent on the concentration of the phorbol ester used (Figs 10 through 13). Although both PdBu and TPA induced potentiation of platelet aggregation in PRP, differences between those two active phorbol esters exist. At equimolar concentrations (60 nmol/L), TPA was more effective than PdBu at inducing a significant aggregation response (30% increase of light transmission at 3 minutes). The potentiation of vasopressin or ADP-induced platelet aggregation (measured after 1 minute) was more pronounced, however, for PdBu than for TPA. The TPA-induced potentiation of platelet aggregation was more evident at later times (≃2 minutes after addition of platelet stimuli) and concerned the extent rather than the velocity of the aggregation response (data not shown).

The length of time of platelet preincubation with PdBu on the potentiation of platelet responses had different effects dependent on the type of agonist used. Upon platelet stimulation with epinephrine, preincubation with PdBu for even 12 minutes did not diminish its synergistic effect on platelet aggregation (Fig 7). In contrast, if vasopressin, ADP or U44069 were used as platelet stimuli, the sensitizing effect of PdBu on platelet aggregation decreased with increasing time of platelet preincubation with PdBu (Figs 8 and 9; data for U44069 are not shown). But even following preincubation of platelets with PdBu for 12 minutes the extent of aggregation induced by low concentrations of vasopressin or U44069 was still higher than in the absence of PdBu (Fig 8). The rate of

Table 1. Rate of Aggregation and Secretion Induced by Vasopressin Plus PdBu is Dependent on the Preincubation Time with PdBu

Stimulus (10 nmol/L)	Time of Preincubation With PdBu	Rate of Aggregation Δ% Light Transmission (per 30 s)	Rate of Secretion ΔμM ATP (per 30 s)
Vasopressin	—*	16	0.03
Vasopressin	5 seconds	20	1.3
Vasopressin	30 seconds	22	1.1
Vasopressin	1 minute	22	0.9
Vasopressin	2 minutes	21	0.9
Vasopressin	4 minutes	20	0.8
Vasopressin	8 minutes	15	0.13
Vasopressin	12 minutes	12	0.10

PRP preincubation with PdBu (100 nmol/L) at 37 °C for the times indicated before addition of vasopressin.

*No PdBu.

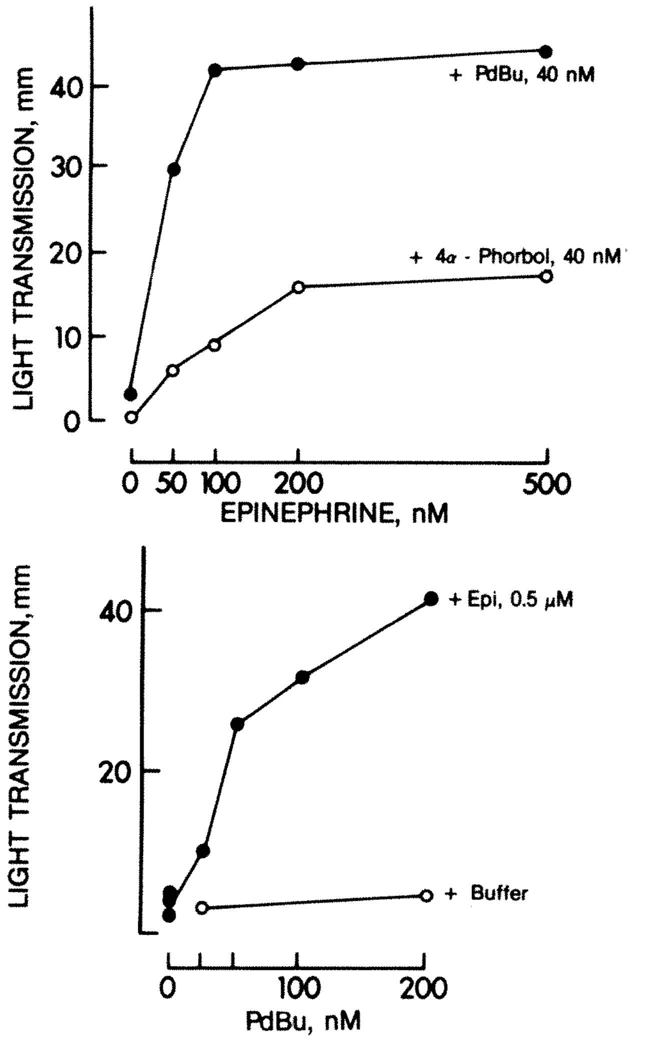

Fig 11. Potentiation of epinephrine-induced platelet aggregation by PdBu. Upper panel: Epinephrine concentration curve. Results obtained with 4α-phorbol were similar to controls. Lower panel: PdBu concentration curve. Both curves were obtained from the same experiment. Conditions were the same as those described in the legend to Fig 10.

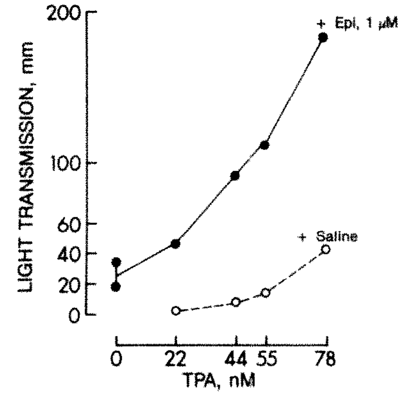

Fig 12. Potentiation of epinephrine-induced platelet aggregation by TPA. Conditions were the same as those described in the legend to Fig 10.

platelet aggregation returned to control levels after prolonged platelet pretreatment with PdBu (Table 1). The potentiation of ATP secretion by PdBu diminished with increasing time of platelet pretreatment with PdBu. The decrease in the secretory response was more rapid than the decrease in aggregation and was observed for all platelet stimuli tested (epinephrine, vasopressin, and U44069).

Studies in washed platelets. Similarly, PdBusensitized platelets toward agonists in suspensions of washed aspirinized platelets suspended in buffer containing 5% PPP and CP/CPK (Figs 8, 9 and 14). The effective concentrations of PdBu (20 to 40 nmol/L) were 5 to 10 times lower than the concentrations needed in PRP. In comparison with the studies in PRP, the sensitizing effect of PdBu disappeared more rapidly in washed platelets with increasing time of PdBu pretreatment. Only upon platelet stimulation with epinephrine was the potentiating effect of PdBu sustained, even if platelets were preincubated for 12 minutes before epinephrine was added (Fig 14). PdBu added to aspirinized as well as to nonaspirinized washed platelets enhanced aggregation and ATP secretion in response to vasopressin, epinephrine, and collagen. PdBu did not, however, increase the stimulus-induced production of arachidonate plus metabolites under these conditions (Table 3).

Protein phosphorylation studies showed that PdBu (20 nmol/L) added to suspensions of washed platelets containing 5% PPP induced only a relatively small activation of protein kinase C. The phosphorylation of the 40-kd protein increased by 20% to 90% over control dependent on the time length of incubation with PdBu (Fig 15). Subsequent addition of vasopressin potentiated protein kinase C activation as reflected by an increase in 40-kd protein phosphorylation (Fig 15). This potentiation was associated with potentiation of platelet aggregation (Fig 15A through C). With increasing preincubation times for PdBu, the potentiation of protein kinase C activation slowed and platelets started to get desensitized to vasopressin (Fig 15D). The potentiation of protein kinase C activation correlated with a reduction of myosin light chain phosphorylation (Fig 15). Vasopressin-induced myosin light chain phosphorylation was increasingly

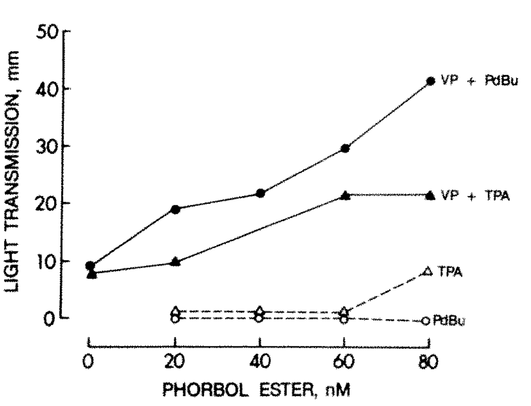

Fig 13. Potentiation of vasopressin (50 nmol/L)-induced platelet aggregation by PdBu or TPA. Aggregation was measured 1 minute after addition of vasopressin. Conditions were the same as those described in the legend to Fig 10.

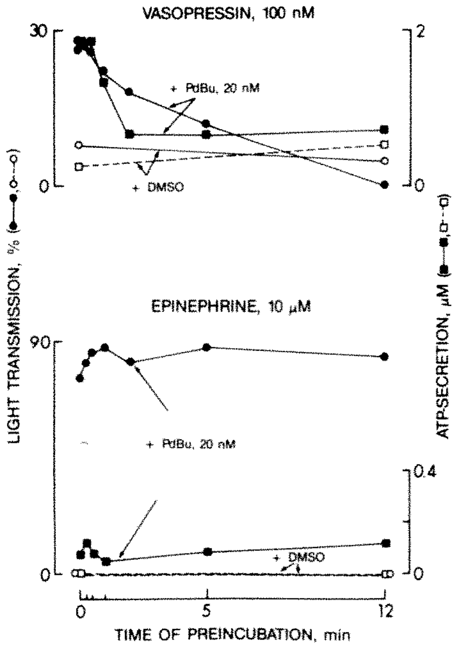

Fig 14. Effect of time of pretreatment with PdBu on responses of washed platelets stimulated by vasopressin or epinephrine.

reduced, if platelets were preincubated for 10 seconds, 1 minute, 5 minutes or 10 minutes with PdBu before addition of vasopressin (Fig 15). These results indicate that potentiation of vasopressin-induced platelet aggregation by PdBu correlated with potentiation of protein kinase C activation and reduction of myosin light chain phosphorylation.

DISCUSSION

Our study indicates that pretreatment of platelets with phorbol esters for various times sensitizes platelets to aggregate and secrete upon stimulation by a wide variety of physiological platelet agonists. This result is surprising, because many studies have demonstrated that phorbol esters desensitize receptor-mediated responses in various cell types, including platelets.[3-12] Most of those studies were performed after the cells were isolated from their physiologic environment and resuspended in an artificial medium. Our study clearly shows that platelets resuspended in plasma show an increased aggregation and secretion response to agonists when they are preincubated with phorbol ester. Enhanced platelet responses could even be observed when the preincubation time with PdBu was extended to 12 minutes. The sensitization became less pronounced by increasing the time of platelet preincubation with PdBu. Using washed platelets resuspended in buffer containing plasma (5%), platelets were also sensitized by pretreatment with phorbol ester, but the sensitizing effect disappeared more rapidly than with PRP.

Our protein phosphorylation studies indicate that the potentiation of platelet aggregation and secretion correlated with a potentiation of protein kinase C activation, but myosin light chain phosphorylation was reduced (Fig 15). These results indicate that protein kinase C stimulation induced during platelet activation is a positive signal for aggregation and secretion, although other proximal biochemical responses such as phospholipase C activation, Ca^{2+} mobilization (data not shown), and myosin light chain phosphorylation (Fig 15) are reduced. It is particularly important for the interpretation of our results that PdBu—although added at relatively high concentrations to PRP (100 or 200 nmol/L) or washed platelets (20 or 40 nmol/L)—induced only a small activation of protein kinase C even after a prolonged preincubation (10 minutes) with platelets. Under our more physiologic experimental conditions (presence of plasma, discoid platelets) protein kinase C in platelets may not be readily accessible to exogenous PdBu. On addition of platelet stim-

Table 3. Effect of PdBu on Stimulus-Induced Production of Arachidonate and Arachidonate Metabolites, Aggregation and ATP Secretion in Control and Aspirinized Platelets

Platelets	Aggregation (mm)	ATP Secretion (μM ATP)	Arachidonate Plus Metabolites in Supernatant (cpm ^{3}H-radioactivity)
Nonaspirinized platelets			
Control	0	0	1884 ± 88 (10)
PdBu 40 nmol/L	0	0	1935 ± 35 (3)
Vasopressin 20 nmol/L	20 ± 0 reversible	0.43 ± 0.03	2028 ± 130 (4)
Vasopressin plus PdBu	87 ± 3	2.21 ± 0.21	1915 ± 61 (3)
Epinephrine, 10 μmol/L	0	0	1912 ± 30 (3)
Collagen 2 μg/mL	80	2.18	4039 ± 250 (3)
Collagen plus PdBu	120	4.37	5137 ± 410 (3)
Aspirinized platelets			
Control	0	0	2490 ± 193 (10)
PdBu 40 nmol/L	5	0.06	2427 ± 17 (3)
Vasopressin 20 nmol/L	40 ± 4 reversible	0.46 ± 0.09	2616 ± 113 (4)
Vasopressin plus PdBu	68 ± 2	2.34 ± 0.16	2463 ± 79 (3)
Epinephrine 10 μmol/L	0	0	2396 ± 90 (3)
Collagen 2 μg/mL	3 ± 2	2.37 ± 0.06	4169 ± 35 (3)
Collagen plus PdBu	18 ± 2	3.18 ± 0.25	4397 ± 319 (3)

Aggregation and ATP secretion were measured after 1 minute; production of AA metabolites was measured 1.5 minutes after addition of platelet stimuli.

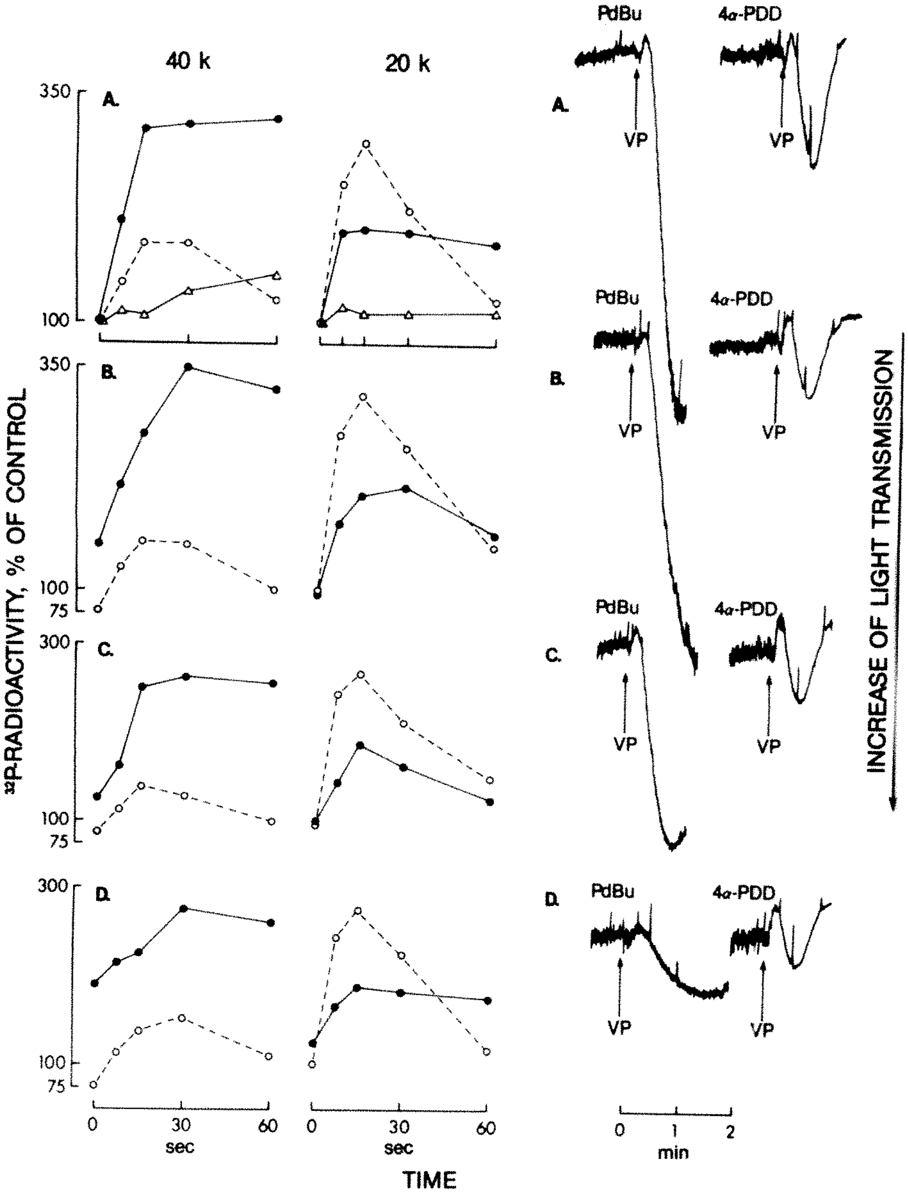

Fig 15. Protein phosphorylation (left) and aggregation tracings (right) of washed platelets pretreated for various time lengths with PdBu (●—●) or 4 α-PDD (○---○) before the addition of vasopressin (VP). Suspensions (0.6 mL) of aspirinized ^{32}P-labeled platelets containing apyrase and 5% plasma were preincubated for 10 seconds (A), 1 minute (B), 5 minutes (C), or 10 minutes (D) at 37°C with 4α-PDD (○---○, 20 nmol/L) or PdBu (●—●, 20 nmol/L) before the addition of VP (50 nmol/L). Stirring was started 30 seconds before addition of VP. Protein phosphorylation (left) was measured in 0.05-mL aliquots of the samples whose aggregation tracings are shown at the right. Protein phosphorylation induced by addition of PdBu alone during stirring is shown in A. The samples containing 4α-PDD gave results identical to those of controls.

uli, however, protein kinase C becomes accessible to phorbol esters. The results further show that small preactivation of protein kinase C is not sufficient to induce desensitization of stimulus-induced functional platelet responses. In previous studies that showed desensitization of stimulus-induced aggregation and secretion by phorbol ester,[4-6,9-11] protein kinase C might have been readily accessible to phorbol esters and maximally activated.

The platelet-aggregating effect of phorbol ester was first described by Zucker et al,[18] who observed, on addition of TPA, a slow, delayed aggregation response of PRP that was not blocked by aspirin or ADP scavengers. We observed that PdBu (100 nmol/L) induced a more significant aggregation response if added to untreated PRP (Table 2) than if added to platelets pretreated with aspirin and PGI_2 and resuspended in plasma in the presence of ADP scavengers (Fig 1). Previous studies clearly show that protein kinase C activation itself does not cause platelet aggregation and secretion.[1,2] In the present study, we observed very little protein kinase C activation by PdBu. Mobilization of membrane-bound Ca^{2+} by phorbol esters, as measured in aequorin-loaded platelets,[19] may be more important for platelet aggregation induced by phorbol esters. Phorbol ester-induced Ca^{2+} mobilization[19] and aggregation but not protein phosphorylation (W. Siess and E.G. Lapetina, unpublished observations) is inhibited by elevation of platelet cyclic AMP levels.

The study shows a clear sensitization of platelet responses to physiologic agonists by phorbol ester. The potentiation of protein kinase C activation may act synergistically with the Ca^{2+} mobilization induced by physiologic agonists and be responsible for the potentiation of aggregation and secretion. Because no attenuation of stimulus-induced functional platelet responses has been found, the desensitization by protein kinase C activation of signal transduction mechanisms (inositol phospholipid hydrolysis, Ca^{2+} mobilization, myosin light chain phosphorylation) might not have been sufficient to be physiologically relevant. On the basis of differences for the various agonists in the dose–response curves and in the decrease of sensitization with increasing time of preincubation of platelets with phorbol ester, two different mechanisms underlying the observed synergism of phorbol ester with platelet stimuli are proposed. One mechanism involves the synergism of phorbol ester with epinephrine, which is not related to potentiation of protein kinase C activation.[20] The other underlies the synergism of phorbol ester with the other agonists, which is related to potentiation of protein kinase C activation. In this regard, it is interesting that epinephrine, in contrast to all other agonists, does not cause phospholipase C activation.[16,21]

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Bone Marrow Transplantation for Leukemia Following a New Busulfan and Cyclophosphamide Regimen

By Peter J. Tutschka, Edward A. Copelan, and John P. Klein

Busulfan 16 mg/kg and cyclophosphamide 120 mg/kg were used as conditioning prior to allogeneic marrow transplantation in 50 adult patients with acute nonlymphocytic leukemia (ANLL), acute lymphocytic leukemia (ALL), and chronic myelogenous leukemia (CML). A standard risk group of 20 patients included those with acute leukemia in remission and CML in chronic phase. A high-risk group of 30 patients included individuals with refractory acute leukemia, acute leukemia in relapse, acute leukemia following preleukemia, and CML in accelerated and blastic phase. Complete remission and sustained complete engraftment were achieved in all evaluable patients. The duration of aplasia was remarkably short (median of 8 days), resulting in a low infection rate during the period of neutropenia, a reduced need for blood product support, and a short length of hospital stay. Three-year actuarial relapse-free survival in both standard-risk (88.9% ± 10.5%) and high-risk (50.5% ± 9.6%) groups compares favorably with that reported with total body irradiation (TBI) containing regimens.

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ALLOGENEIC BONE MARROW transplantation following total body irradiation (TBI) and high-dose chemotherapy has provided encouraging therapeutic results in patients with hematologic malignancies.[1] Apparent cure of $\sim$50% of patients with acute nonlymphocytic leukemia (ANLL)[2] in first remission and chronic myelogenous leukemia (CML) in chronic phase has been achieved[3]; the success rate in acute lymphocytic leukemia (ALL) has been somewhat lower.[4] Despite these results, several undesirable features of allogeneic marrow transplantation have limited its application.[5] First the usual side effects of the conditioning regimen together with prolonged periods of isolation often make transplantation a difficult ordeal for the patient. Second, common complications of marrow transplantation such as infections, graft-*v*-host disease (GVHD) and interstitial pneumonia (IP) may be lethal. Third, chronic complications such as chronic GVHD may result in a substandard quality of life despite a "successful" outcome. Last, relapse occurs in 10% to 40% of individuals transplanted under the best circumstances. These problems have understandably dampened the enthusiasm for this therapy.

The plateau in survival rates, observed over the recent past, may in large part reflect limitations of the traditional regimen of TBI and cyclophosphamide (Cy). Past attempts to replace TBI with chemotherapeutic agents have not met with an increased success rate.[6] In a previous trial using busulfan with higher doses of Cy than are commonly used with TBI, a significantly lower relapse rate for ANLL in remission was achieved. A higher complication rate, however, caused a similar overall long-term disease-free survival as compared with TBI-containing regimens.[7]

Because busulfan is the primary antileukemic agent in our regimen and because of the high complication rate in the previous trial, we used as a new conditioning regimen the same dose of busulfan but a lower dose of Cy. We describe the activity of this regimen in 50 consecutive adult patients transplanted with CML in chronic, accelerated, or blastic phase, and with ALL and ANLL in first or second remission, in relapse, or with refractory disease.

MATERIALS AND METHODS

All patients were entered on a study protocol that had been reviewed and approved by The Ohio State University Institutional Review Board.

Patient accrual. Fifty adult patients were consecutively entered into the study. Eligibility for the study required a diagnosis of ALL, ANLL, or CML, regardless of the remission status. All patients were ambulatory, had an HLA-identical sibling donor, and were aged >15 and ≤ 50 years. No other medical parameters were used as exclusion criteria. All diagnoses of leukemia were confirmed by examination of the bone marrow aspirate and biopsy. On admission, all patients had a bone marrow aspirate and bone marrow biopsy as well as a diagnostic lumbar puncture to assess the status of the leukemia. Presence of leukemic cells in the spinal fluid was not an exclusion criterion. All data are analyzed as of February 14, 1987. The patients were transplanted betwen February 13, 1984 and August 14, 1986; therefore, the observation period for these patients has been a minimum of 6 months and a maximum of 3 years.

Study groups. The study involved two groups of patients. A standard-risk group consisted of patients with acute leukemia in the first or second remission, or CML in the chronic phase. Twenty patients, 11 with ANLL in remission, 2 with ALL in remission, and 7 with CML in chronic phase were in this group.

The high-risk group consisted of 30 patients with refractory acute leukemia, acute leukemia in relapse, or CML in accelerated or blastic phase. Refractory acute leukemia was defined as leukemia that failed to remit after two courses of conventional chemotherapy. Patients were considered to be in the accelerated phase of CML if they had $\geq 10\%$ blasts in blood or marrow together with major perturbations of the leukocyte or platelet count unresponsive to chemotherapy with busulfan or hydroxuyrea. They were considered to be in the blastic phase if they had $\geq 30\%$ blasts in the blood or bone marrow. Among the patients in the high-risk group were two previously untreated patients in whom leukemia developed after a preleukemic syndrome (UPN 4 and UPN 17).

Twelve patients had ANLL, 4 had ALL, and 14 patients had CML in accelerated ($n = 9$), or blastic phase ($n = 5$).

Patient criteria. The median age of the recipients was 28 years (range 15 to 50 years). The median age of patients in the standard-risk group was 32 years (range 19 to 50 years), and the median age of

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the high-risk group 27 years (range 15 to 48 years). Thirty-four patients were male and 16 patients were female. Thirty-four patients were sex matched with their recipients (20 male to male, 9 female to female transplants), and 16 patients were sex mismatched (6 male to female and 10 female to male transplants). All patients were identical for A, B, C, D/Dr locus with their allogeneic sibling donors. When possible, genotypical analysis of the entire family was performed. Seven patients had major blood group mismatches with their donors. The erythrocytes were removed from the marrow inoculum of these donors by centrifugation prior to the marrow infusion.[8]

Bone marrow donors. All donors were required to give informed consent prior to the procedure. All donations were carried out under general anesthesia. The technical aspects of the procedure have been described elsewhere.[9] All donors tolerated the marrow aspiration well and were discharged between 1 and 2 days after the marrow harvest. No significant complications of the marrow harvest procedure occurred.

Preparative regimen. All patients were given methotrexate intrathecally (10 mg/m^2 of body surface but not more than 12 mg total) ~10 days prior to marrow transplantation. Three patients who had central nervous system (CNS) leukemia at the beginning of the transplant procedure were given four intrathecal treatments of methotrexate. Patients received busulfan orally at a dose of 1 mg/kg of ideal body weight four times daily for 4 consecutive days for a total dose of 16 mg/kg. Cyclophosphamide at a daily dose of 60 mg/kg of ideal body weight was administered intravenously (IV) over a 1-hour period for 2 consecutive days. Bone marrow was infused 2 days after the final dose of Cy.

Supportive therapy. On the day of hospitalization, all patients were placed in ultraclean rooms equipped with HEPA-filtered laminar air flow. Patients were treated in strict isolation. "Sterile diet" with very low bacterial content was begun on day −4 together with oral Clotrimazole and oral Trimethoprim-Sulfamethoxazole. Nonabsorbable antibacterial antibiotics were not used. All patients had a double-lumen and a single-lumen right atrial catheter placed. All patients received IV a commercial immunoglobulin preparation (Sandoglobulin®) at a dose of 500 mg/kg every 2 weeks starting 1 week prior to transplant and ending at day 120 after transplant. The first 31 patients did not receive immunoglobulin after that time, but the 19 most recently transplanted patients were given the same immunoglobulin preparation monthly until day 365. In an attempt to prevent hemorrhagic viral gastroenteritis, the same immunoglobulin preparation was given orally at a daily dose of 50 mg/kg in four divided doses, starting at day 2 after transplant and maintained until day 28.[10] All blood products administered were derived from volunteer donors who had a negative antibody titer against cytomegalovirus.

Transplantation and evaluation of engraftment. The bone marrow was infused on day 8 through a right atrial catheter over 4 hours. All patients tolerated the infusion well. The mean number of nucleated marrow cells infused was $5.17 \times 10^8 \pm 0.25$ (±1 SE)/kg body weight of the recipient. This number was not corrected for peripheral blood contamination. Engraftment was assessed indirectly by peripheral blood counts and periodic marrow examinations. An average of four routine marrow examinations were carried out for each transplant course, the last one performed between 120 and 160 days (mean 147 days) after transplant. After that time, bone marrow examinations were only carried out if hematologic and cytogenetic parameters measured in the peripheral blood suggested the recurrence of leukemia. Engraftment was measured directly by cytogenetic markers, when available (sex or autosomal markers), RBC enzymes, and RBC antigens.

Determination of remission status posttransplantation. Patients were considered to be in complete remission (CR) after transplantation for acute leukemia if they showed a completely normal bone marrow and peripheral blood status together with the absence of lymphohematopoietic cells of recipient origin by cytogenetic analysis.

Patients were considered to be in complete remission after transplantation for CML if they showed a completely normal bone marrow and peripheral blood status together with the absence of lymphohematopoietic cells of recipient origin by cytogenetic analysis. Patients who showed the presence of the Philadelphia chromosome prior to transplantation required the complete and persistent disappearance of this chromosomal abnormality after transplantation to qualify for complete remission status.

GVHD. All patients received a combination of cyclosporine and methylprednisolone to prevent GVHD. Cyclosporine started 1 day prior to transplantation and was given as a continuous infusion of 5 mg/kg daily for 4 days. After that time, the dose of cyclosporine was reduced to 3 mg/kg given IV over 6 hours daily until day 14. From day 14 through day 35, the dose of cyclosporine was increased to 3.75 mg/kg given IV. Thereafter, cyclosporine was slowly tapered until day 180, after which time it was discontinued. At discharge, patients were given oral cyclosporine at a dose four times higher than the IV dose. Methylprednisolone was started on day 7 at a dose of 0.5 mg/kg/day. On day 14, the dose was increased to 1 mg/kg/day and was given until day 28. Then, until day 72, the methylprednisolone was slowly tapered. All patients received prophylaxis for CNS leukemia. Between day 70 and day 90 after bone marrow transplant, four intrathecal doses of 10 mg/m^2 each of methotrexate were administered over 2 to 4 weeks.

Acute GVHD was assessed according to the grading system described in the literature.[11] Chronic GVHD was assessed using descriptive criteria reported previously. Patients were assumed to have chronic GVHD if they had the typical features of the disease[12] and/or were given adrenal steroids after day 100. Patients with chronic GVHD were divided into three groups according to severity, grade 1 disease showing mild symptoms that resolve within 2 months, grade 2 showing mild symptoms that require 2 to 6 months for resolution without sequelae, and grade 3 disease showing marked symptoms that require >6 months of therapy.

Other common complications. Interstitial pneumonia was diagnosed when bilateral interstitial/alveolar infiltrates on chest x-ray were associated with hypoxemia (PO_2 on room air of ≤65 mm Hg). Hemorrhagic gastroenteritis was diagnosed when diarrhea of greater than 1,000 mL/day was present that was either bloody or maroon and persisted for >3 days.

Statistical analysis. Estimates of survival probabilities and the probability of being in remission were computed by using the product-limit estimates of Kaplan and Meier.[13] In estimating the probability of being in remission, we treated those patients who died free of leukemic disease as censored observations. A Cox's proportional-hazards analysis was performed in each of the clinical variables displayed in Tables 1 and 2. Significance was judged using the maximum likelihood chi-squared statistic.[14]

RESULTS

Early toxicity of the preparative regimen. The preparative regimen was generally very well tolerated. Mild nausea and vomiting were noted during busulfan administration in nine patients; the remaining patients all tolerated busulfan well. On the days during which Cyclophosphamide was administered, most patients experienced nausea and vomiting requiring antiemetic therapy. None of the patients developed severe oral mucositis, but most patients complained of mild sore throat between 7 and 11 days after

Table 1. Cytogenetic Analysis of Cells in Peripheral Blood

Time	No. of Samples	No. of Patients	Median Day	No. of Donor Cells	Total Cells	Donor Cells (%)	SE
0-20	26	22	17	438	513	85.4	1.56
21-40	32	24	29	403	514	78.4	1.81
41-70	35	23	60	537	634	84.7	1.43
71-100	36	25	84	707	777	91.0	1.02
101-160	25	21	130	620	665	93.2	0.97
161-220	18	15	184	395	404	97.8	0.73
221-365	29	18	284	608	613	99.2	0.36
>365	25	9	593	491	491	100.0	0.00

transplant. Of the 50 patients, 32 had no evidence of hemorrhagic cystitis. Eight showed minimal microscopic hematuria, 5 patients showed macroscopic hematuria, and 5 patients showed severe hemorrhagic cystitis. Clinical symptoms of venoocclusive disease occurred in one patient (UPN 37), but resolved without any sequelae.

Antileukemic effect. With the exception of one patient who died on day 2 (UPN 38), all patients experienced a complete remission. This includes all patients with refractory disease and those in blast crisis of CML.

None of the patients transplanted in the standard-risk group to date have shown evidence of relapse. Among the 30 patients in the high-risk group, five patients all transplanted for acute leukemia experienced a clinical relapse (UPN 2, 7, 22, 35, and 52). Of the 14 patients transplanted for CML in accelerated or blastic phase, only one patient has shown evidence of relapse by cytogenetic analysis (UPN 21). This patient, who developed recurrence of the Philadelphia chromosome in the marrow, was retransplanted using the same conditioning regimen. She is alive, disease-free 777+ days after the first transplant and 185+ days after the second transplant. The remaining 13 patients transplanted for CML in the accelerated or blastic phase to date remain in complete remission with a normal bone marrow and peripheral blood status, absence of the Philadelphia chromosome, and presence of lymphohematopoietic cells of exclusively donor type. A Kaplan-Meier product limit estimate for the probability of being in remission is shown in Fig 1.

Engraftment and marrow function. One patient who died on day 2 from cardiac failure (UPN 38) could not be evaluated for engraftment. One other patient (UPN 71) showed a recovery marrow on day 14, but never reached a WBC that exceeded $1.0 \times 10^9/L$ or a platelet count of $40 \times 10^9/L$. The remaining 48 patients all showed complete and sustained engraftment. The patients maintained a WBC level $>1.0 \times 10^9/L$ for a median of 5 days after marrow infusion, after which time most patients' counts fell precipitously. At a median of 13 days (range 10 through 35) they achieved a WBC level $>0.5 \times 10^9/L$ and a level $>1.0 \times 10^9/L$ at a median of 15 days (range 11 through 36). The median time to reach a self-sustaining platelet count of $40 \times 10^9/L$ was 19 days (range 11 through 38). Bone marrow biopsies obtained on day 14 after transplant showed the presence of all hemapoietic cell lines, including megakaryocytes in all patients except the one patient who could not be evaluated for engraftment (UPN 38).

Table 2. Clinical Parameters Associated With Survival (Cox-Hazards)

Parameter	*P* Value	SE	*P* Value	Relative Risk
Refractoriness	1.79	0.69	0.0034	5.98
Interstitial pneumonia	1.28	0.319	0.0001	3.60
Acute GVHD (≥grade 2)	2.61	0.872	0.0028	13.59
Hemorrhagic cystitis	2.584	0.68	0.0001	13.316

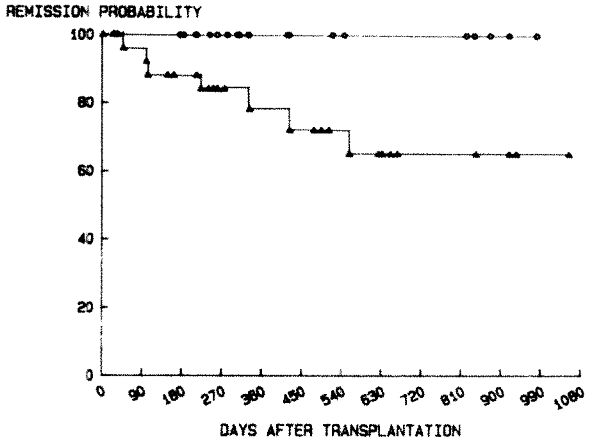

Fig 1. Probability of being in remission for patients transplanted in the standard-risk group *v* the high-risk group. Standard risk 100%, —○—; high risk 65.5% ± 11.1 (SE), —△—.

The degree of chimerism was assessed primarily by cytogenetic analysis. Cytogenetic analysis of the bone marrow was performed with each bone marrow aspirate. Cytogenetic analysis of the peripheral blood was performed every 2 weeks during the first 3 months and thereafter at each visit of the patient to the transplant center.

At day 14 after transplant, all patients had a marrow aspirate performed. Twenty-three patients showed technically sufficient cytogenetic preparations (14 sex markers and 9 autosomal markers) with a mean of 12.6 metaphases examined per patient. All cells identified cytogenetically at day 14 were of donor origin.

At least one second bone marrow examination was carried out, the latest performed at a mean of 123 days after transplantation. Twenty-six patients showed technically sufficient cytogenetic preparations with a mean of 18.4 metaphases examined per patient. Of these 26 patients, 24 had exclusively donor cells in their marrow. One patient (UPN 66) showed two of ten metaphases to be of recipient type at 71 days after transplant. He remains in complete remission on day 243+ after transplantation. One other patient (UPN 13) showed 1 of 17 cells to be of recipient type on day 446 after transplant. This patient, transplanted for CML in blast crisis, remains alive in complete remission on day 919+ after transplantation.

The cytogenetic analysis of the peripheral blood cells can be summarized as follows. Two hundred twenty-six samples were taken from 34 patients or an average of 6.64 samples per patient. The median number of samples per patient was 6.5 (range 1 to 15). Four thousand six hundred eleven cells were examined, an average of 20.40 per sample or 135.61 per patient. The number of cells per patient examined ranged from 13 (1 determination) to 390 (15 determinations). The median number of cells per patient examined was 115.5. The median number of cells per sample was 20 (range 1 to 50). The mode was 32. The median day of these examinations was 87.5 (range 12 to 767). The interquartile range was 117.5. Four thousand one hundred ninety-nine (4,199) of the cells examined were classified as donor cells (91.06% with an SE of .42%).

During the first 20 days, 85.4% of the cells were classified as donor. With time, the number of donor cells increased steadily until by day 365 after transplant only donor cells could be identified in the peripheral blood. A breakdown of the quantitative analysis over time is given in Table 1.

Support during the first 30 days after transplantation. Due to the brief period of aplasia, relatively few blood products were required to maintain a hemoglobin of ≥10 g/dL and a platelet count of ≥20 × 10^9/L. Patients transplanted in the high-risk group were given a median of 5 U RBCs (range 0 through 42) and five transfusions of single donor platelets (range 0 through 137). The patients transplanted in the standard-risk group received a median of 3 U RBCs (range 0 through 10) and a median of four transfusions of platelets (range 0 through 20). Eleven high-risk and eight standard-risk patients (38%) did not develop a temperature >100.5°F in the first 30 days and required no therapeutic antibiotics. Only one patient was given amphotericin-B for a fungal infection (UPN 71). One patient was documented to have bacteremia with *Pseudomonas aeruginosa* (UPN 43), probably derived from a cutaneous *Pseudomonas* abscess that antedated the transplant procedure. This patient responded promptly to appropriate antibiotic therapy. Two patients developed stomatitis with herpes simplex virus, which responded to Acyclovir. Three patients developed *Candida* esophagitis documented by endoscopy. All three responded promptly to oral nonabsorbable antifungal agents. Patients were discharged from the hospital when their granulocyte count was >0.5 × 10^9/L on three successive days and platelet and RBC counts were self-sustaining. Furthermore, they were required to be afebrile, off therapeutic antibiotics, and no longer receiving parenteral nutrition. The median day of discharge of all patients was 22 days (range 18 to 71), of the patients in the standard-risk group it was 21 days (range 18 to 33), and of the patients in the high-risk group it was 26 days (range 19 to 71).

GVHD. Acute GVHD was present in 15 patients. Of these fifteen patients, eleven showed grade 1 disease, one showed grade 2 disease, two showed grade 3 disease, and one showed grade 4 disease. All three patients with severe GVHD (grades 3 and 4) died in part from the GVHD. Chronic GVHD of grade 1 occurred in 5 patients, chronic GVHD of grade 2 occurred in 15 patients, and chronic GVHD of grade 3 occurred in 5 patients. Chronic GVHD resolved with no sequelae in all but two patients. Chronic GVHD was associated with death in 2 patients with grade 3 disease. Chronic GVHD has been completely absent to date in 16 of the 35 surviving patients. Of the 45 patients surviving 100 days, 19 or 42% have shown clinically significant chronic GVHD of grade 2 or greater at any time during their transplant.

Interstitial pneumonia and hemorrhagic gastroenteritis. Interstitial pneumonia occurred in six patients, four of whom died with the disease. In 2 patients cytomegalovirus could be identified by bronchoalveolar lavage, 2 patients had idiopathic interstitial pneumonia, and 1 patient each had pneumonia associated with *Legionella* and *Pneumoncystis carinii*. Two patients developed hemorrhagic gastroenteritis. In one of the two patients, hemorrhagic gastroenteritis was associated with severe GVHD, of which the patient died. The other patient recovered.

Late infections. Late infections (past day 50) occurred in 13 patients. Two patients developed systemic fungal infections (*Candida albicans*, UPN 24; *Aspergillus fumigatus*, UPN 19), and 12 patients developed bacterial infections, all with gram-positive organisms. Of these 12 bacterial infections, 8 were caused by *Streptococcus pneumoniae*, and 4 by *Staphylococcus epidermidis*. All bacterial infections presented with fever and minimal associated clinical symptoms and had documented bacteremia. Most bacterial infections (11 of 12) occurred after day 120 and have not been seen after IV immunoglobulin therapy was extended to 1 year posttransplant.

Survival. Of the 50 patients transplanted, 35 remain alive in unmaintained clinical remission. The actuarial 3-year survival rate of this patient population is 65%. The survival curves for all patients transplanted are shown in Figs 2 and 3. All patients in the standard-risk group transplanted for either ANLL or CML are disease-free survivors, and only 1 of the 20 patients transplanted in the standard-risk group (for ALL, UPN 19) has died. This death was associated with chronic GVHD and systemic *Aspergillus* infection, the patient being in complete remission at the time of death.

Of the 30 patients transplanted in the high-risk group, 16 are alive at present in unmaintained clinical remission. Nine of the 14 patients transplanted for CML in accelerated or blastic phase are alive, and 7 of the 16 patients transplanted for advanced acute leukemia are alive in unmaintained complete remission. The survival curves for these patient groups are shown in Figs 4 and 5.

Parameters affecting survival. Parameters that might

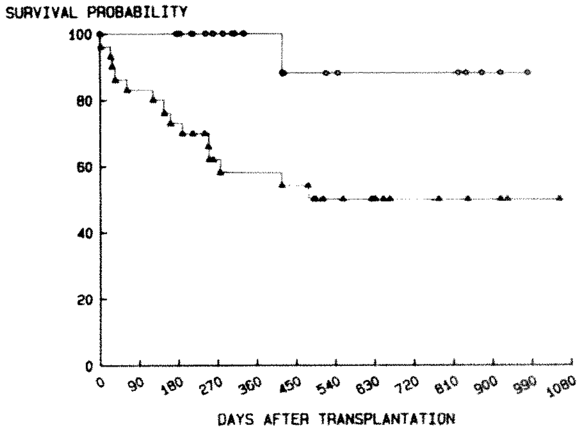

Fig 2. Probability of survival for patients transplanted in the standard-risk group v the high-risk group. Standard risk 88.9% ± 10.5 (SE), —O—; high risk 50.6% ± 9.6 (SE), —△—.

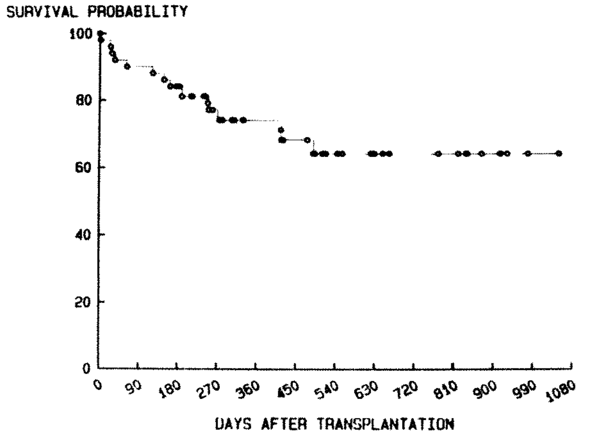

Fig 3. Probability of survival of all patients transplanted. Survival 64.9% ± 7.6 (SE), —O—.

affect survival were entered into a Cox regression proportional hazards model for death as outcome variable. The parameters included: diagnosis; disease status; risk group; age and sex of the recipient; time of return of blood counts; day of discharge; number of late infections; and the complications of hemorrhagic cystitis, hemorrhagic gastroenteritis, interstitial pneumonia, acute GVHD, and chronic GVHD. Refractory disease, interstitial pneumonia, hemorrhagic cystitis, and acute GVHD of grade 2 or greater were all significantly associated with death. These data are shown in Table 2.

DISCUSSION

Busulfan and Cy are an attractive combination for pretransplant conditioning. Preclinical studies in a rat model have demonstrated the importance of myelosuppression in combination with immunosuppression for preparing marrow graft recipients.[15] In this model, the addition of busulfan, a nonimmunosuppressive but highly myelotoxic agent, to Cy, a moderately myelotoxic but highly immunosuppressive agent, permitted the reduction of Cy without compromising engraftment.[16] Previous clinical trials using a combination of busulfan and high-dose Cy demonstrated excellent antileukemic efficacy, but serious toxicity similar to TBI-containing regimens.[6,7] The present study attempted to show that the reduction of Cy would not compromise antileukemic effectiveness of the combination, but would markedly reduce the complication rate. The reduction of Cy to 120 mg/kg, the commonly used dose in TBI-containing regimens, permitted furthermore the direct comparison of busulfan to TBI in the hope of achieving overall a lower complication rate.

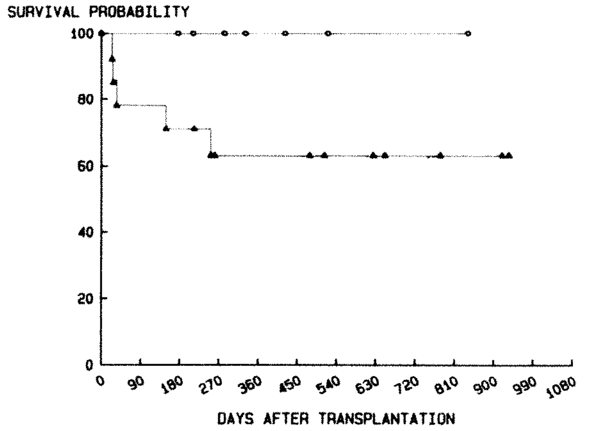

Fig 4. Probability of survival for patients transplanted for CML in the standard-risk group *v* the high-risk group. Standard risk 100%, —O—; high risk 63.5% ± 13.0 (SE), —△—.

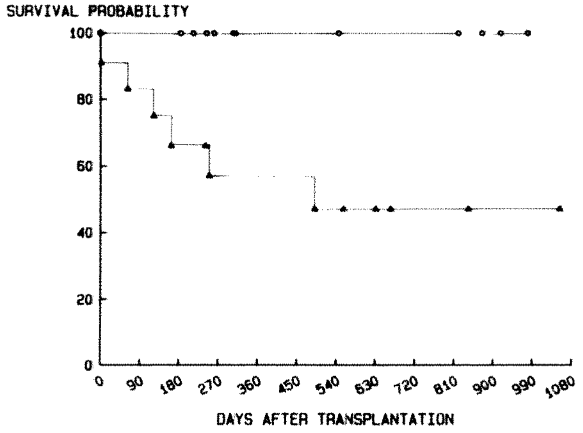

Fig 5. Probability of survival for patients transplanted for ANLL in the standard-risk group *v* the high-risk group. Standard risk 100%, —O—; high risk 47.6% ± 15 (SE), —△—.

The data presented in this report bear out these predictions. The preparative therapy was well tolerated, and apart from moderate yet transient nausea and vomiting associated with Cy, no immediate toxicity occurred. Apart from one patient who died too early for evaluation, all patients, including those with refractory leukemia or in blastic phase of CML, achieved complete remissions and complete sustained engraftment. As predicted from the animal model, marrow recovery occurred rapidly. Fully functioning hematopoietic grafts were usually achieved within the first 2 weeks after transplantation. It is tempting to speculate that the busulfan, by virtue of its "space making" properties, was responsible for this short aplasia.[6,16] It is equally possible, on the one hand, that the avoidance of TBI was less damaging to the microenvironment, permitting earlier engraftment. On the other hand, substituting cyclosporine and methylprednisolone for methotrexate in the prevention of GVHD might have reduced the induced aplasia after transplantation. This short period of aplasia resulted in a markedly reduced need for blood product support and, most important, in a surprisingly low incidence of infectious complications. There was a virtual absence of bacteremia (one patient) and only one patient required amphotericin-B administration. Other common complications, such as acute GVHD were also infrequent. The low incidence of severe acute GVHD may have resulted from prophylaxis with a new combination regimen of cyclosporine/methylprednisolone. The low incidence of early infectious complications resulting from short aplasia, lesser amounts of tissue toxicity, and ultraclean environmental conditions may have led to a reduced incidence of

GVHD, a postulate supported by a number of preclinical and clinical observations.[17,18]

It is unclear to what extent the reduced incidence of interstitial pneumonia can be credited to the preparative regimen. Because radiotherapy and acute GVHD contribute to the development of interstitial pneumonia,[19] the low incidence in our study is probably not just a spurious observation. Furthermore, the patients received IV immunoglobulin preparations and blood products derived from cytomegalovirus-antibody–negative donors, both procedures reportedly associated with a lower incidence of viral interstitial pneumonia.[20-22]

Late complications seen after allogeneic bone marrow transplantation were not altered in frequency when compared with TBI-containing regimens. Some patients developed gram-positive bacteremias after 100 days, none of which led to the death of the patients. These often quite insidious infections have not been observed since the administration of IV immunoglobulin was extended to 1 year posttransplant.

Similarly, chronic GVHD occurred frequently. Fortunately, this complication has been very mild and in most patients chronic GVHD has resolved completely without sequelae.

The antileukemic efficacy of this regimen is apparent. Although the disease-free survival in regular-risk patients so far is outstanding, clearly additional follow-up time is required to ascertain that these patients are indeed cured. The excellent results achieved in advanced disease demonstrate a potent antileukemic effect, however. The results in accelerated and blastic phase of CML compare favorably to published results in recent studies that use TBI and Cy.[3]

The patients studied here were at high risk for several reasons. They were older than patients in most comparable studies, some of our patients being 50 years old. Older age clearly increases the transplant risk.[2,3,23] Many had advanced disease, including high-risk factors such as pretransplant hepatic dysfunction, cytogenetic abnormalities, CNS disease, and cutaneous involvement, all parameters associated with poor prognosis.

The results of this study are encouraging. This regimen may be more effective and less toxic than TBI-containing regimens for patients generally considered to be good transplant candidates, eg, those with acute leukemia in remission and CML in chronic phase. In addition, this regimen offers promise in circumstances such as CML in accelerated or blastic phase and in refractory acute leukemia in which transplantation results have been so poor that valid questions as to the appropriateness of allogeneic marrow transplantation have been raised. Furthermore, the low toxicity of this regimen might permit the use of additional antileukemic agents in patients with advanced disease.

This report suggests that this new regimen provides an alternative to traditional conditioning regimens that include TBI. It offers substantial promise in decreasing the morbidity and improving the outcome of patients undergoing allogeneic marrow transplantation.

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We thank the nursing staff of the Bone Marrow Transplant Unit and The Ohio State University Housestaff for their outstanding efforts. We are indebted to all referring physicians; to Dr Leona Ayers, head of Microbiology; to Phyllis Kaldor, head nurse of the Bone Marrow Transplant Unit; to Linda K. Graff, Transplantation Coordinator; to Becky Gibbins, Clinical Protocol Nurse; and Judy Bishop, Data Coordinator, for their many contributions. We thank Judy French and Barbara Fowls for preparing the manuscript and for numerous other contributions.

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Effect of Oxalate and Malonate on Red Cell Metabolism

By Ernest Beutler, Linda Forman, and Carol West

The addition of oxalate to blood stored in Citrate-phosphate-dextrose (CPD) produces a marked improvement in 2,3-diphosphoglycerate (2,3-DPG) preservation; an increase in 2,3-DPG levels can also be documented in short-term incubation studies. Oxalate is a potent in vitro inhibitor of red cell lactate dehydrogenase, monophosphoglycerate mutase, and pyruvate kinase (PK). In the presence of fructose 1,6-diphosphate the latter inhibitory effect is competitive with phospho(enol)pyruvate (PEP). Determination of the levels of intermediate compounds in red cells incubated with oxalate suggest the presence of inhibition at the PK step, indicating that this is the site of oxalate action. Apparent inhibition at the glyceraldehyde phosphate dehydrogenase step is apparently due to an increase in the NADH/NAD ratio. Oxalate had no effect on the in vivo viability of rabbit red cells stored in CPD preservatives for 21 days. Greater understanding of the toxicity of oxalate is required before it can be considered suitable as a component of preservative media, but appreciation of the mechanism by which it affects 2,3-DPG levels may be important in design of other blood additives. Malonate, the 3-carbon dicarboxylic acid analogue of oxalate did not inhibit pyruvate kinase nor affect 2,3-DPG levels.

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OVER A DECADE AGO it was demonstrated that ascorbic acid exerted a favorable effect on the 2,3-diphosphoglycerate (2,3-DPG) level of erythrocytes in stored blood.[1,2] Subsequent studies revealed that ascorbic phosphate, a more stable derivative of ascorbic acid had a similar effect.[3] Although this phenomenon has been confirmed repeatedly,[4-6] our investigation of the mechanism of the ascorbate effect failed to provide an explanation for the increase in 2,3-DPG that occurred.[1,7,8] Specifically, we could not find significant inhibition of glycolytic enzymes by ascorbate,[1] no evidence of oxidation of either NADH or NADPH, and no evidence that the ascorbate was metabolized or that it exerted a significant effect on intracellular pH.[7]

Recently, Kandler et al[9] found that in contrast to the usual preparations, highly purified ascorbic acid and ascorbic phosphate had no effect on the 2,3-DPG of stored red cells. Searching for a contaminant that might explain the effect of less purified batches, they discovered that oxalate found in the ascorbate preparations could account for the effect on the 2,3-DPG levels of red cells in stored blood.

We now report on the effects of oxalate on the metabolism of human erythrocytes, identifying some of the inhibitory actions that may explain the effect of this compound on 2,3-DPG levels. We have also explored the effect of malonic acid, a natural metabolic intermediate and the 3-carbon analogue of the 2-carbon dicarboxylic acid, oxalic acid, on 2,3-DPG levels.

MATERIALS AND METHODS

After obtaining informed consent, blood samples from normal blood donors were drawn into citrate-phosphate-dextrose (CPD), for storage studies at 4°C or into 1 mg/mL ethylenediamine tetracetic acid (EDTA) for short-term incubations at 37°C. Potassium oxalate and sodium malonate were purchased from Sigma Chemical Company, St. Louis.

Unless otherwise indicated red cell enzyme activities were measured using standard methods,[10] adding the stated amount of oxalate to the assay system. Pyruvate kinase (PK) activity was estimated using a system in which the generation of adenosine triphosphate (ATP) is measured in order to circumvent the inhibitory effect of oxalate on the lactate dehydrogenase reaction used as an indicator in the standard method. In this technique, the reaction mixture contained the following: 100 mmol/L Tris-HCl, pH 8; 0.5 mmol/L EDTA; 10 mmol/L $MgCl_2$, 100 mmol/L KCl; 0.2 mmol/L NADP; 2 mmol/L glucose; 1.5 mmol/L adenosine diphosphate (ADP); 0.1 U each of glucose-6-phosphate (G-6-P) dehydrogenase and hexokinase. The reaction was started by the addition of phospho(enol)pyruvate (PEP) to provide a concentration of 5 mmol/L. The effect of oxalate on the kinetics of the PK reaction were studied using partially purified PK.[11] Diphosphoglycerate phosphatase (DPGP) activity was measured by estimating inorganic phosphate (P_i) release from labeled 2,3-DPG.[12]

ATP, 2,3-DPG, and pyruvate and lactate levels were measured spectrophotometrically as previously described.[10] G-6-P, fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (GAP), 3-phosphoglyceric acid (3-PGA), 2-phosphoglyceric acid (2-PGA), and PEP were all measured fluorometrically.[10] In some instances, the levels of DHAP, GAP, and FDP were sufficiently elevated in the erythrocytes so that their levels could also be measured by a spectrophotometric modification of the standard fluorometric technique. The internal pH of stored red cells was measured at 4°C using a Corning model 150 with a glass electrode, packing the erythrocytes by centrifugation, freezing, and thawing. Red cell viability was studied in New Zealand white rabbits using the combined ^{99m}Tc-^{51}Cr method.[13]

RESULTS

Cold storage of CPD anticoagulated blood with and without oxalate. Four hundred fifty milliliters of blood

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from each of four normal donors was collected into 63 mL of CPD and into CPD containing sufficient potassium oxalate to provide a final concentration of 500 μmol/L. The blood was stored at 4°C, mixed at intervals, and the levels of ATP and 2,3-DPG were determined. The results of these investigations are shown in Fig 1. There was no difference in internal red cell pH for the first 2 weeks of storage. At 3 weeks the internal pH of the samples with oxalate averaged 0.061 U higher and at 4 weeks 0.11 U higher than the controls. The initial determination of red cell ATP and 2,3-DPG levels was made after the anticoagulated blood had been held at room temperature for approximately two hours. It was apparent that ATP levels in blood collected into oxalate-containing anticoagulants had already declined substantially in this brief time. This immediate effect of oxalate on red cell ATP levels was confirmed in other studies (data not shown). Over the 4-week storage period oxalate greatly improved preservation of 2,3-DPG but had a markedly deleterious effect on ATP levels. The same experiment was carried out with a final oxalate concentration of only 50 μmol/L. The results of this study are shown in Fig. 2.

The effect of oxalate on red cell enzyme activities. The effect of 500 μmol/L potassium oxalate on red cell enzyme activities is summarized in Fig 3. The enzyme most sensitive to inhibition by oxalate was found to be lactate dehydrogenase (LDH), followed closely by monophosphoglyceromutase (MPGM) and then by PK. In contrast to the effect of oxalate, 5 mmol/L malonate failed to inhibit erythrocyte MPGM, PK, or LDH activity. The effect of oxalate on PK kinetics is shown in Fig 4. The inhibition of PK by oxalate was competitive with PEP in the presence of FDP, the allosteric effector of PK, but noncompetitive in the absence of FDP.

The effect of oxalate on red cell metabolism at 37°C. Estimation of the levels of metabolic intermediates other than ATP and 2,3-DPG at the end of the 28-day storage period proved to be unreliable because of the very large amounts of pyruvate and lactate that had accumulated.

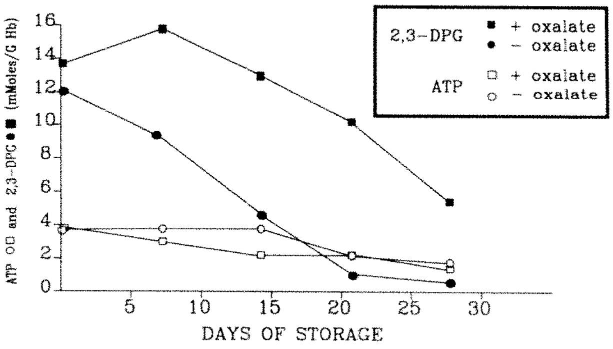

Fig 2. The same as Fig 1, except that the oxalate concentration was only 50 μmol/L.

However, studies of the effect of oxalate on red cell intermediates could readily be carried out on red cells incubated in autologous plasma for several hours at 37°C, since a rise in the red cell 2,3-DPG level was demonstrable under these conditions. It was necessary to use an anticoagulant other than CPD for such studies, because of the very substantial effect that temperature exerts on the pH of blood. It is ~0.7 pH units higher at 4°C than at 37°C[14] and the citrate preservatives provide an unsuitable medium for study of metabolism at the latter temperature. A suspension of red cells in EDTA anticoagulated plasma incubated under a suitable CO_2-air atmosphere is very suitable for such investigations. Preliminary studies showed that maximum elevation of red cell 2,3-DPG levels could be observed after four to six hours incubation at 37°C and that the amount of phosphate in plasma was insufficient to support an optimal effect of oxalate on 2,3-DPG concentration.

Erythrocytes from blood drawn into EDTA anticoagulant were resuspended at a hematocrit of 30% in their own plasma. Sufficient KH_2PO_4/K_2HPO_4 pH 7.4 was added to provide a 2.5 mmol/L increase in PO_4 concentration. Aliquots of the cell suspension were incubated at 37°C in a shaking incubator in an atmosphere of 5% CO_2/95% air with

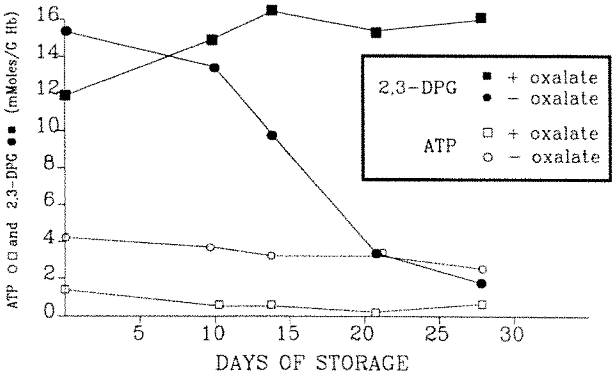

Fig 1. 2,3-DPG and ATP levels of blood stored in CPD (○, ●) and CPD plus sufficient oxalate to provide a final concentration of 500 μmol/L (□, ■). Four 450 mL units of blood were studied. Each point represents the mean of two observations. The 0 time points were obtained after a one to two hour holding period at room temperature. Following this the samples were stored at 4°C and mixed only at the time of sampling.

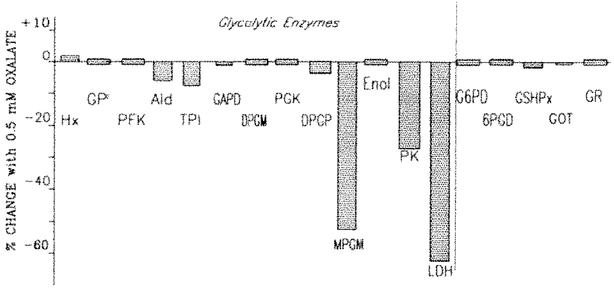

Fig 3. The effect of 500 μmol/L oxalate on the activity of some red cell enzymes. Abbreviations: Hx, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase, ALD, aldolase, TPI, triose phosphate isomerase; GAPD, glyceraldehyde phosphate dehydrogenase; DPGM, diphosphoglycerate mutase; PGK, phosphoglycerate kinase; MPGM, monophosphoglycerate mutase; Enol, enolase; LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GSHPx, glutathione peroxidase; GOT, glutamate oxalocetate transaminase; GR, glutatione reductase.

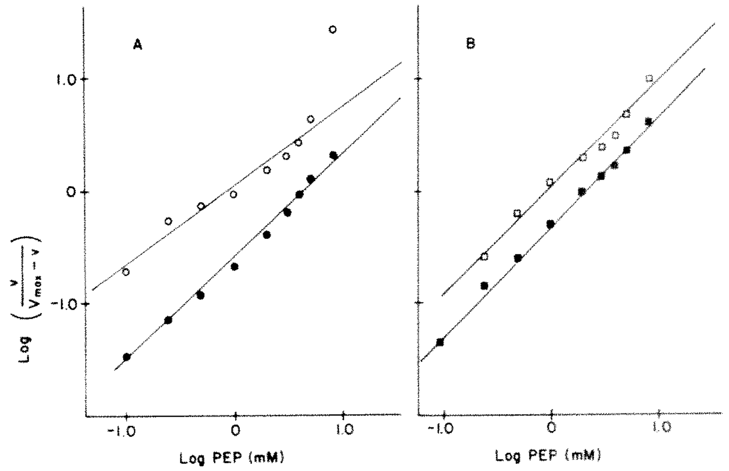

Fig 4. The kinetics of the inhibition of PK by 500 μmol/L oxalate. Hill plots are presented (A) in the presence of 500 μmol/L FDP, and (B) in the absence of FDP. PK was purified using standard methods.[11] The inhibition by oxalate was competitive with respect to PEP in the presence of FDP and noncompetitive in its absence.

and without the addition of 2 mmol/L oxalate. The possible limiting effect of phosphate and of NAD^+ was investigated in incubation mixtures to which excess phosphate or pyruvate had been added. The pH and the levels of metabolic intermediates are presented in Table 1. Figure 5 represents a "cross-over" plot[15] based on these data. In a similar short-term incubation experiment malonate failed to exert any effect on the level of 2,3-DPG.

The effect of oxalate on red cell survival. Twenty milliliters of rabbit blood was collected into 2.8 mL of CPD solution and divided into bags to which no oxalate was added and those to which sufficient potassium oxalate was added to provide a final concentration of 500 μmol/L. At the end of 21 days storage the survival of the red cells of one of the samples was measured in a normal recipient rabbit, and at the end of 22 days the other sample was labeled with a larger amount of ^{51}Cr and viability again determined in a rabbit. The order of presentation was varied so that two of the rabbits received red cells that had been stored with oxalate on day 21 and the other two those that had been stored with oxalate on day 22. The results of these studies are presented in Table 2. Shown also is the effect of storage of rabbit blood on red cell ATP and 2,3-DPG levels. Oxalate exerted the same favorable effect on 2,3-DPG concentrations and unfavorable effect on ATP concentration of rabbit erythrocytes as were observed with human cells.

DISCUSSION

Although oxalate has long been used as an anticoagulant for the collection of blood for diagnostic studies, no thought has apparently been given to its use for blood storage. This has probably been due to the presumed toxicity of oxalate. However, the concentration of oxalate required to aid in the maintenance of red cell 2,3-DPG levels is much lower than that required as an anticoagulant. Even a concentration of only 50 μmol/L exerted a substantial favorable affect on red cell 2,3-DPG: after 21 days' storage the levels had declined by only about one-third. Assuming uniform distribution throughout body water, the instant administration of 450 mL of blood containing this concentration of oxalate would result in a rise of only ~0.6 micromolar in the oxalate level, an increase of the order of 20%. One must take into account, of course, the effect of the administration of multiple units of blood, the rate of clearance of oxalate in normal and diseased individuals, and the existence in the population of many individuals who may have hereditary abnormalities in oxalate transport[16] potentially leading to calcium oxalate stone formation.

Table 1. Levels of Metabolic Intermediates in Red Cell Suspensions Incubated With and Without Oxalate and the Indicated Additives

	2.5 mmol/L Phosphate		10 mmol/L Phosphate		2.5 mmol/L Phosphate 10 mmol/L Pyruvate	
	Control	Oxalate	Control	Oxalate	Control	Oxalate
Glucose-6-phosphate (nmol/mL)	63.78	24.84	65.93	23.60	52.84	29.65
Fructose-6-phosphate (nmol/mL)	8.68	9.80	29.67	16.85	25.36	15.63
Fructose-1-6-diphosphate (nmol/mL)	2.55	14.56	11.5	325.4	2.25	3.79
Dihydroxyacetone-phosphate (nmol/mL)	31.38	779.83	36	1084	11.71	46.56
Glyceraldehyde-3-phosphate	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2,3 Diphosphoglycerate (μmol/gHb)	13.82	18.27	11.45	13.26	14.35	18.72
3-Phosphoglyceric acid (nmol/mL)	98.65	560.84	81.1	556.38	94.70	448.55
2-Phosphoglyceric acid (nmol/mL)	8.46	36.24	7.99	33.23	N.D.	N.D.
Phosphoenolpyruvate (nmol/mL)	15.86	161.66	7.53	205.6	19.9	154.9
Pyruvate (μmol/L)	328.9	46.50	254.4	20.03	7984.2	14055.0
Lactate (μmol/L)	7320.65	8532.15	7460.9	8522.5	7147.0	6788.1
pH at t = 6 hr	7.506	7.498	7.668	7.614	7.543	7.696

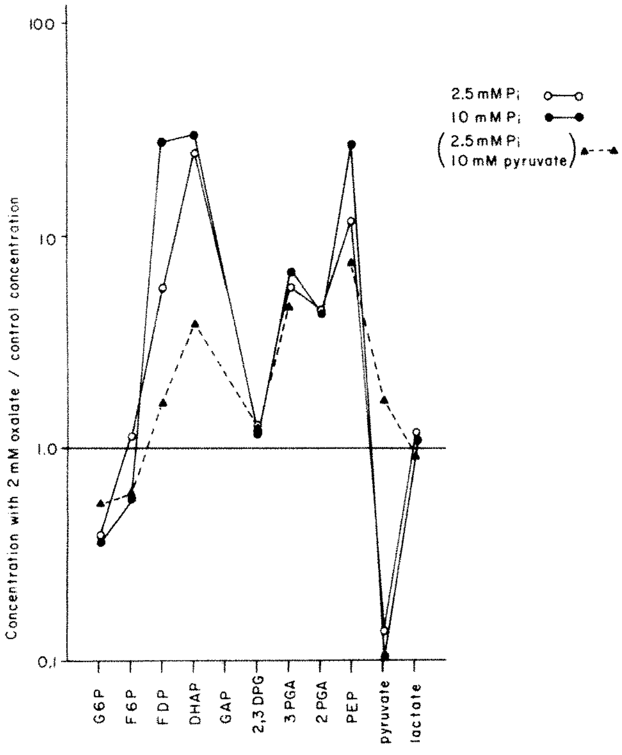

Fig 5. A crossover plot demonstrating the effect of oxalate after six hours' incubation in autologous plasma at 37°C under 95% air-5% CO_2. The effect of added P_i and pyruvate are shown: concentrations represent the amount added to those already present in plasma.

The discovery that oxalate increases the levels of 2,3-DPG in stored blood provides a solution to the problem of why ascorbate appeared to have this effect. We are unable to explain why ascorbate preparations failed, in our earlier studies,[1] to result in enzyme inhibition. It is possible that the preparation used in the enzyme assays was of higher purity than those used in the storage studies. Since these investigations were carried out some 15 years ago, the purity of the various lots used can no longer be determined. Similar differences in purity probably account for the discrepancy between our finding that dehydroascorbate was ineffective[7] while Dawson et al[4] subsequently reported that it was more effective than was ascorbate itself.

Efforts to maintain 2,3-DPG during prolonged blood storage are generally directed at creating appropriate modifications of the metabolic flow within the erythrocyte. Such a modification may consist of providing an additional substrate for red cell metabolism. Dihydroxyacetone and inosine are such substances. Dihydroxyacetone is converted to DHAP by red cell triokinase.[17] Inosine undergoes phosphorolysis to ribose-1-phosphate and hypoxanthine, and the ribose-1-phosphate is converted in the hexose monophosphate pathway to F-6-P and to GAP.[18] In each of these cases use of the new substrate is less sensitive to a fall in the pH of stored blood than is glucose, providing a distinct metabolic advantage to the stored erythrocytes. An alternative to providing a more efficient substrate is modifying red cell preservatives so as to inhibit or to stimulate reactions that influence 2,3-DPG synthesis or degradation. In the past, manipulations of hydrogen ion, pyruvate, and phosphate concentrations have been used for this purpose.[19] Increasing the pH stimulates the diphosphoglycerate mutase (DPGM) and inhibits the DPGP reactions.[20] Pyruvate serves to oxidize NADH to NAD, thereby increasing the level of one of the required co-factors for the glyceraldehyde phosphate dehydrogenase (GAPD) reaction.[21,22] The product of this reaction, 1,3-DPG is the immediate substrate for the formation of 2,3-DPG. Phosphate stimulates glycolysis, probably largely by relieving the inhibition of hexokinase by G-6-P.[23]

Table 2. The Effect of Oxalate on the Storage of Rabbit Blood

	ATP (μmol/g Hb)		2,3-DPG (μmol/g Hb)		Viability (%)
Storage Medium	Day 0	Day 21	Day 0	Day 21	Days 21-22
CPD*	7.34	1.76	28.65	13.12	71.2, 68.8, 65.6, 64.4
CPD + oxalate	7.34	0.72	28.65	28.12	72.6, 78.5, 66.8, 69.6

*The same animals served as controls in a concurrent investigation of BW440C.[12]

It is the DPGM and DPGP reactions that directly control the level of 2,3-DPG in erythrocytes, but these reactions, in turn, are influenced by other metabolic changes in the erythrocyte. PK deficiency is associated classically with increased 2,3-DPG levels, sometimes even in the heterozygous state.[24] The effect of PK deficiency is presumably due to the accumulation of substrates proximal to the PK reaction, ie, PEP, 2-PGA, and 3-PGA. The latter compound inhibits DPGP and stimulates DPGM. Vora recently suggested that inhibitors of this reaction might be useful in maintaining 2,3-DPG levels during blood storage.[25]

In the present investigation we extended the observations of Kandler et al.[9] Red cell membranes are readily permeable to the oxalate anion,[26] even at 0°C.[16] Oxalate increases 2,3-DPG levels not only in blood stored in an artificial additive solution, but also in CPD anticoagulant. Although ATP depletion accompanied the improved 2,3-DPG maintenance, red cell viability was not impaired, at least in rabbits. Oxalate did not exert its effect by altering intracellular pH. It is a known inhibitor of both plasma LDH[27] and of L type (red cell) PK.[28] We found that not only these enzymes but also red cell monophosphoglycerate mutase is inhibited by oxalate. Our kinetic studies indicated that the inhibition of PK is competitive with respect to PEP when the allosteric effector, FDP is present, a finding that is consistent with data obtained earlier in the study of L type PK.[28]

At 37°C oxalate increases the 2,3-DPG levels of red cells incubated in their own plasma even for a few hours. This provided us with the opportunity of addressing the question of how oxalate accomplishes its effect on 2,3-DPG levels. The studies in whole red cells pin-point the major functional lesion induced by oxalate to the PK reaction: the cross-over plot (Fig 5) shows that the concentration of intermediate compounds proximal to the PK reaction is increased and that those distal to the reaction are decreased. However, a second cross-over was also present at the GAPD step. A deficiency of either phosphate or NAD could produce such an effect. The cross-over before GAPD is not eliminated by increasing the phosphate concentration by 7.5 mmol/L. However, increasing the available NAD by the addition of pyruvate[21,22] greatly decreased the accumulation of substrates proximal to the GAPD reaction. Thus, it appears that in addition to its effect on the PK reaction, oxalate produces a change in the NADH/NAD ratio of red cells, possibly merely by inhibiting the LDH reaction.

Although the potential toxicity of oxalate might preclude its clinical use, its effect suggests that other PK inhibitors might be useful, if one that was sufficiently nontoxic could be found. It is of interest, in this respect that malonate, the structure of which superficially resembles PEP more closely than does that of oxalate, has no effect on either the PK reaction or on 2,3-DPG levels in incubated red cell suspension. Oxalate is a normal plasma constituent, circulating at a level of ~4 μmol/L.[29,30] It may be that this compound may exert some in vivo regulatory effect, and further studies are underway to explore this possibility.

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Intensive Chemotherapy in Aggressive Lymphomas: Updated Results of LNH-80 Protocol and Prognostic Factors Affecting Response and Survival

By Bertrand Coiffier, Paul-André Bryon, Martine Ffrench, Michel Blanc, Catherine Sebban,
Françoise Berger, and Jean-Jacques Viala

One hundred patients with aggressive malignant lymphomas treated with the LNH-80 regimen were evaluated for long-term survival and pretreatment characteristics predictive of response and survival. LNH-80 consists of three intensive courses of adriamycin cyclophosphamide viudesine bleomycin (ACVB) followed by sequential consolidation and final intensification. Eighty-four patients went into complete remission (CR), eight had a partial response (PR), three failed to respond, and five died during induction. Twenty-three patients (27%) relapsed, in two of whom a prolonged second remission was obtained. Sixty-three patients are currently alive, two of them with disease. Four patients died in CR. Median survival and median freedom from relapse survival were not reached with a median follow-up of 4½ years. Characteristics negatively associated with response in multivariate analysis were: poor performance status, bone marrow involvement, and two or more extranodal sites of disease. Duration of CR was associated with splenic involvement. Three characteristics were negatively associated with survival in multivariate analysis: age, high grade subtypes, and bone marrow involvement.

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THE POOR PROGNOSIS associated with aggressive lymphomas has been dramatically improved by intensive combination chemotherapy. Regimens such as M-BACOD,[1] ProMACE-MOPP,[2] MACOP-B,[3] and LNH-80[4] have been associated with increases of 10% to 30% in complete remission (CR) rates over those attained by CHOP regimens,[5,8] and a concurrent decrease in relapse rates. Thus a greater number of patients survive 3 years of more. However, the long-term results of these intensive regimens are not known and their superiority is not definitively accepted. Multicenter randomized studies are currently in progress in the United States and Europe to verify the better results of intensive regimens over those obtained with CHOP regimens. Even with these newer regimens, there are some patients who fail to respond or who relapse prematurely. Prognostic factors have been identified in patients treated with CHOP[5,9] or newer[10] regimens. This report updates the results of the LNH-80 regimen on 100 patients, with a median follow-up of 4½ years. Results already reported after 2 years of follow-up[4] have been maintained with a plateau over 60%. Factors associated with response and/or survival are specified.

PATIENTS AND METHODS

Patients. One hundred patients with aggressive malignant lymphoma (ML) were treated with the LNH-80 regimen: the 97 patients previously described[4] and three additional patients who had been excluded from the first publication due to the long time interval between diagnosis and treatment. Since they did not receive any chemotherapy or radiotherapy during this interval, they were brought into this study. All the patients were treated in our department between March 1980 and April 1984. All patients had given their informed consent in accordance with local institution guidelines. Entrance criteria for the protocol have been previously described[4]: diagnosis of aggressive ML according to the Working Formulation,[11,12] stage II or greater for intermediate grade ML, all stages for high grade ML, age <75 years, no contraindication to adriamycin, no previous chemotherapy or radiotherapy.

Of the 100 patients, 69 were males and 31 were females. Median age was 48.5 years (range 11 to 73 years, with three patients <15 and eight patients >65 years). Initial staging procedures included chest radiography, bone marrow aspiration and biopsy, liver function tests, CSF examination, abdominal echography and/or computed tomography (CT). Other examinations were performed as clinically indicated. The clinical presentation was detailed in the earlier report.[4] Briefly, 66 patients had stage IV disease, 48 had B symptoms, 46 a performance status >1, 65 a large tumoral mass, 21 one extranodal site. Lactate dehydrogenase (LDH) was ≥ 2.5 times the normal level in 29 patients. Bone marrow involvement was present in 34 patients. Morphological subtypes are listed in Table 1.

Treatment. The LNH-80 regimen shown in Fig 1 included three phases of chemotherapy. The induction phase consisted of three courses of ACVB. A fourth course was administered if CR had not been obtained after three courses. The courses were initiated every 15 days or when polymorphonuclears increased to $>1{,}500 \times 10^9$/L. A consolidation phase of sequential chemotherapy was initiated 1 month after the last ACVB. A final intensification was started 2 months later with two courses of AraCVmB. CNS radiotherapy was administered in cases of initial CNS localization.

Prognostic factors. The factors evaluated for potential prognostic significance included sex, age, performance status, presence of B symptoms, morphologic subtype, immunologic phenotype, sites of involvement. number of extranodal sites of disease, bulky (more than 7 cm in diameter) tumor, serum LDH, alkaline phosphatase and protein levels, complete blood count, and intensity of administered drugs during the induction phase. Performance status was based on the Eastern Cooperative Oncology Group scale: 0 to 4. Pathologic review was done by two hematopathologists (PAB and FB) according to the Working Formulation.[11,12] Immunologic phenotypes were determined in 37 patients by light chain characterization or monoclonal antibody typing,[13] either on cell suspensions or on frozen specimens. LDH levels were expressed as multiples of the maximum normal value. Intensity of administered drugs during the induction phase was calculated as the number of milligrams per

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Table 1. Results of Treating 100 Patients With the LNH-80 Regimen, According to Histologic Subtypes Defined in the Working Formulation

Histologic Subtypes	N	CR	Relapse	Percent Surviving at 5 y ± SE	No. at Risk
Intermediate grade (%)	47	38 (81)	9 (24)	72 ± 7	29
Follicular large cell	3	2	—	66 ± 27	2
Diffuse mixed	26	21	4	68 ± 9	14
Diffuse large cell	18	15	5	74 ± 10	14
High grade (%)	48	41 (85)	13 (32)	52 ± 7	21
Immunoblastic	23	19	5	46 ± 11	8
Lymphoblastic	6	6	2	67 ± 19	4
Small noncleaved	19	16	6	53 ± 11	10
Histiocytic	2	2	—	100	2
Unclassifiable	3	3	2	33 ± 27	1
Total (%)	100	84 (84)	23 (29)	61 ± 5	45

There is no statistical difference between intermediate and high-grade lymphomas for response and relapse rates ($P > .10$) and for survival duration ($P > .05$).

square meter of cyclophosphamide and adriamycin per 15 days and expressed as a percentage of the theoretical dose.[14]

Assessment of response. Response to treatment was assessed at the start of the consolidation phase. CR was defined by the disappearance of all clinical evidence of disease and the normalization of all laboratory values. Some patients with persisting radiographic abnormalities were deemed to be in CR if morphologic examination did not reveal any evidence of lymphoma: these patients were considered to have a persisting necrotic mass.[15] Partial response (PR) was defined as >50% reduction in the largest dimension of each site of measurable disease. The failure category comprised progressive disease, transient disease regression, or death during the induction phase.

Statistical methods. Duration of remission was calculated from the onset of the consolidation phase to the first objective evidence of relapse. Survival duration was calculated from the beginning of treatment until death. Survival curves were estimated by the method of Kaplan and Meier.[16] Univariate analyses were performed using the χ^2 and log-rank tests.[17] Any factor significant at the 0.10 level was included in a logistic regression analysis of response[18] and in a proportional hazards model regression analysis of survival[19] using the BMDP package (Statistical Sofware, Los Angeles).

RESULTS

Eighty-four patients (84%) achieved CR. Five patients (5%) died during the induction phase, eight patients (8%) presented a PR, and three patients (3%) failed to respond. Two of the eight PR patients were alive at 2 years, but none were alive at 4 years. Of the 84 CR patients, 23 (27%) relapsed 5 to 56 months after diagnosis. Only six of the patients relapsed after 18 months, and two more of them relapsed after 3 years. CNS relapse was observed in two of the ten patients with initial CNS localization, but in none of the others. Results according to histologic subtype and immunologic phenotype are presented in Tables 1 and 2. Nineteen of the 23 patients who relapsed were rebiopsed: A histologic progression was seen in five patients and a follicular pattern was seen in two.

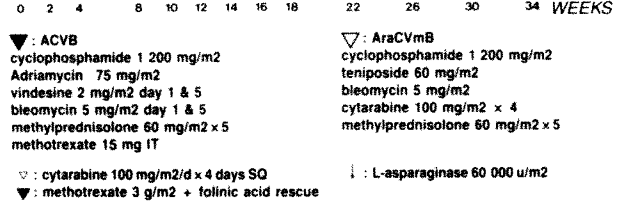
INDUCTION
CONSOLIDATION
FINAL INTENSIFICATION
0
2
4
8
10
12
14
16
18
22
26
30
34
WEEKS
▼: ACVB
cyclophosphamide 1 200 mg/m2
Adriamycin 75 mg/m2
vindesine 2 mg/m2 day 1 & 5
bleomycin 5 mg/m2 day 1 & 5
methylprednisolone 60 mg/m2 x 5
methotrexate 15 mg IT
▽: AraCVmB
cyclophosphamide 1 200 mg/m2
teniposide 60 mg/m2
bleomycin 5 mg/m2
cytarabine 100 mg/m2 x 4
methylprednisolone 60 mg/m2 x 5
▿ : cytarabine 100 mg/m2/d x 4 days SQ
▾ : methotrexate 3 g/m2 + folinic acid rescue
↓ : L-asparaginase 60 000 u/m2

Fig 1. Scheme of the LNH-80 regimen.

Sixty-three patients (63%) are currently alive, two with disease and two in a stable second CR of >3 years. Four patients died in CR: one case each of CMV encephalitis, interstitial pneumonia, staphylococcus septicemia, and acute myeloblastic leukemia (18 months after diagnosis). Overall treatment mortality rate was 9% (five patients during induction and four in CR). Overall survival and freedom from relapse (FFR) survival are presented in Fig 2. Median survival and median FFR survival were not reached with a median follow-up of 4½ years.

Prognostic indicators of response to chemotherapy. Forty-five clinical or biological factors determined at diagnosis were evaluated individually as possible prognostic indicators of response to therapy. The response rates associated with some of these factors are shown in Table 3. Statistically significant ($P < .05$) factors negatively associated with response were age >50 years, poor performance status, bulky tumor, two or more extranodal sites of involvement, bone marrow, hepatic, splenic, or pleural involvement, hemoglobin <100 g/L and LDH >2.5 times the normal level.

Single factors not predictive of response to therapy

Table 2. Results According to Immunologic Phenotypes of the 37 Phenotype Patients Treated With the LNH-80 Regimen

Immunologic Phenotype	N	CR	Relapse	Percent Surviving at 5 y ± SE	No. at Risk
B cell	24	20	9	61 ± 10	10
Peripheral T cell	13	9	2	35 ± 14	3

There is no statistical difference in response and relapse rates between B cell and peripheral T cell lymphomas. There is a borderline significant difference for overall survival (Mantel-Cox-Savage test: $P = .05$).

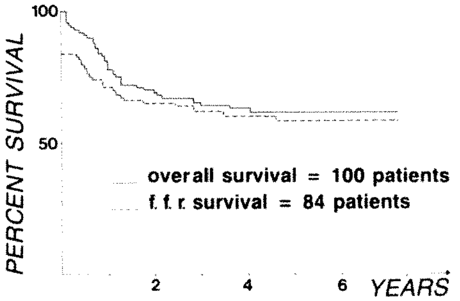

Fig 2. Overall survival and freedom from relapse survival of 100 patients with aggressive lymphoma treated with the LNH-80 regimen.

Table 3. Response Rates According to Some Initial Factors of Patients Treated With LNH-80 Regimen

	N	CR	PR	Failure	P Value
Entire group	100	84	8	8	
Age					
<50 y	52	48	1	3	
≥50 y	48	36	7	5	.003
Performance status					
0-1	54	52	2	—	
≥2	46	32	6	8	.003
B symptoms					
A	52	48	3	1	
B	48	36	5	7	.076
Weight loss					
No	67	60	5	2	
Yes	33	24	3	6	.06
Stage					
I-II	23	23	—	—	
III	11	11	—	—	
IV	66	50	8	8	.13
Tumoral mass					
None	35	32	2	1	
One	55	46	6	3	
Several	10	6	—	4	.004
No. of extranodal sites of disease					
0	20	20	—	—	
1	21	21	—	—	
≥2	59	43	8	8	.04
Bone marrow involvement					
Absent	66	62	2	2	
Present	34	22	6	6	.003
Hepatic involvement					
Absent	65	60	4	1	
Present	35	24	4	7	.005
Splenic involvement					
Absent	66	59	3	4	
Present	34	25	5	4	.02
Pleural involvement					
Absent	86	75	7	4	
Present	14	9	1	4	.015
LDH level					
<2.5 × normal	66	61	3	2	
≥2.5 × normal	29	19	5	5	.01
Hemoglobin level					
<100 g/L	14	8	3	3	
≥100 gr/L	84	74	5	5	.04
M/m-BACOD score					
1	19	19	—	—	
2	24	24	—	—	
3	57	71	8	8	.03

P values are those given by the χ^2 test.

included sex, morphologic subtype, immunologic phenotype, stage, B symptoms; digestive, head and neck, cutaneous, pulmonary, pericardiac, osseus, or neurologic localization; presence of a large mediastinal or abdominal mass; decreased level of proteins, albumin, gammaglobulins, platelets, or polymorphonuclear leukocytes; increased level of gammaglobulins, lymphocytes, lymphoblasts, or creatinine; accelerated sedimentation rate, and drug intensity of induction chemotherapy.

The factors associated with response according to univariate unadjusted analysis were further examined by multivariate logistic regression analysis. All the factors having prognostic significance were known for all the patients except the LDH level for five patients. Two studies were performed: the first on 95 patients with all the factors, the second on all the patients without the LDH level. The two studies gave the same results: poor performance status, bone marrow involvement, and two or more extranodal sites remained statistically significant ($P < .05$).

None of the factors studied was statistically associated with relapse in univariate or multivariate analyses. Drug intensity during the induction phase had borderline significance ($P = .07$). The presence of a residual necrotic mass in 16 patients was not associated with a greater risk of relapse ($P = .15$).

Prognostic indicators of survival. The clinical and biological factors examined for their value in predicting response were also assessed for their relation to survival (Table 4). Single factors statistically associated with short survival were bone marrow involvement ($P < .01$) (Fig 3), LDH >2.5 times the normal level ($P = .02$), age >50 years ($P = .02$), hemoglobin level <100 g/L ($P = .03$), and more than one extranodal site ($P = .05$). Poor performance status and peripheral T cell phenotype had borderline significance: P value < 0.05 in the Wilcoxon test, and <0.10 in the Mantel-Cox-Savage test. None of the other factors tested was statistically associated with poor survival, particularly age, morphologic subtype, stage, B symptoms, hepatic, splenic, digestive, or neurologic localization, and drug intensity during the induction phase.

All the factors with some significance were included in a multivariate Cox regression analysis without the stepwise selection (Table 5). The analysis was performed on two sets of patients: all 100 patients, and the 95 for whom LDH was known. Factors found to be significantly associated with poor survival were increasing age, high grade subtypes, and bone marrow involvement.

The prognostic score described by Shipp et al[10] in their study with the M/m-BACOD regimens was applied to our patients. This score was based on the performance status, the diameter of the largest mass, and the number of extranodal sites of disease; it divided patients into three subgroups with a predicted 5-year survival rate of 68%, 55%, and 24%. Figure 4 shows the Kaplan-Meier survival curves for our

Table 4. Estimates of Survival Probability of Patients Treated With the LNH-80 Regimen: Kaplan-Meier Analysis for Some Initial Factors and for Shipp's Score Based on Patients Treated by the M/m-BACOD Regimens[10]

	N	Percent Surviving at 5 y ± SE	No. at Risk	*P* Value
Entire group	100	61 ± 5	45	
Age				
<50 y	52	73 ± 6	35	
≥50 y	48	48 ± 5	15	.02
Performance status				
0-1	54	68 ± 7	29	
≥2	46	54 ± 7	24	.06
Symptoms				
A	52	62 ± 7	24	
B	48	60 ± 7	28	.54
Weight loss				
No	67	60 ± 6	28	
Yes	33	64 ± 8	21	.92
Tumoral mass				
None	35	68 ± 8	21	
One	55	59 ± 7	24	
Several	10	50 ± 16	5	.14
No. of extranodal sites				
0	20	80 ± 9	15	
1	21	71 ± 10	14	
≥2	59	52 ± 7	21	.05
Bone marrow involvement				
Absent	66	69 ± 6	34	
Present	34	47 ± 9	15	.02
Hepatic involvement				
Absent	65	66 ± 6	37	
Present	35	53 ± 9	12	.15
Splenic involvement				
Absent	66	66 ± 6	40	
Present	34	50 ± 10	8	.14
Pleural involvement				
Absent	86	61 ± 5	39	
Present	14	64 ± 13	9	.95
LDH level				
<2.5 × normal	66	67 ± 6	33	
≥2.5 × normal	29	48 ± 9	14	.02
Hemoglobin level				
<100 g/L	14	43 ± 13	6	
≥100 g/L	84	66 ± 5	43	.03
M/m-BACOD score				
1	19	78 ± 10	13	
2	24	71 ± 9	17	
3	57	51 ± 7	18	.05

P values are those found in the Mantel-Cox-Savage test.

patients divided into Shipp's risk groups. We obtained predicted 5-year survival rates of 78%, 71%, and 51% (Table 4). The score was a statistically significant indicator of prognosis: *P* value < 0.05 for response and survival. In multivariate analyses, the score did not remain a significant predictor of the probability of response, relapse or death.

DISCUSSION

Sixty-four patients (64%) with aggressive ML treated by the LNH-80 regimen are alive, 61 of them in persistent CR with a median follow-up of 4½ years. These results are superior to those obtained with CHOP and CHOP-like regimens.[5-8] A large proportion of patients will probably be cured, but 3 to 5 more years are required before the results can be considered definitve. These results are the best reported with a follow-up >3 years: the M/m-BACOD regimens have achieved a median survival of 68 months with a 5-year survival rate of 50% for large cell ML[10]; the ProMACE-MOPP/CytaBOM regimens and the MACOP-B regimen have not been updated as yet, but the MACOP-B regimen was reported to give good results only in large cell ML.[20]

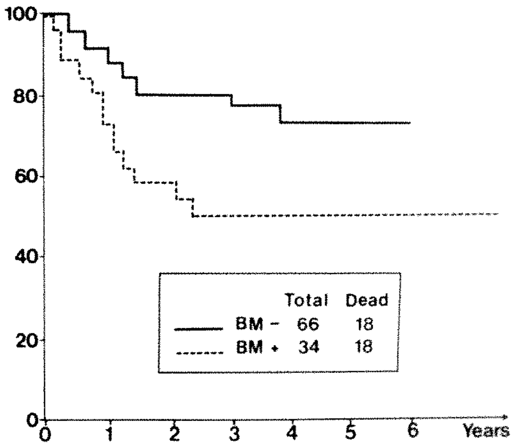

Fig 3. Effect of bone marrow involvement on the survival of patients with aggressive lymphoma treated with the LNH-80 regimen.

Our 100 patients were evaluated for prognostic factors that could be predictive of response and survival. Poor performance status, bone marrow involvement, and two or more extranodal sites of disease were found to be negatively associated with response. Age, high grade subtypes, and bone marrow involvement were adversely associated with survival. Marrow involvement was one of the most important prognostic factors, as had also been reported for the CHOP regimen,[5,7,9,21,22] but not in the M/m-BACOD study.[10]

The prognostic factors we found to be significant had been reported in several previous studies on prognostic factors in ML. Poor performance status and presence of B symptoms had been recorded as indicators of low response rate and/or poor survival.[5,7,9,10,22,23] Age was considered an indicator of poor survival in most of the studies, but the dividing line was variable: 55 years,[22] 55 and 65 years,[23] 60 years.[24] Visceral localizations as liver, spleen, pleura were occasionally asso-

Table 5. Multivariate Proportional Hazards Regression Analysis of Survival of Patients Treated With the LNH-80 Regimen

	Analysis of Survival	
Prognostic Factors	Estimated Coefficient	Significance Level
Age (y)	0.0390	.001
Morphology (intermediate, high)	1.0067	.012
Bone marrow involvement (no, yes)	0.7974	.032
Performance status (0-1, ≥2)	0.5569	.187
LDH (x normal)	0.1506	.325
No. of extranodal sites (0, 1, ≥2)	0.1893	.502

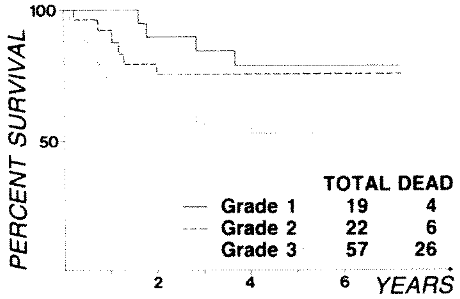

Fig 4. Survival of patients with aggressive lymphoma treated with the LNH-80 regimen according to the score described by MA Shipp based on patients treated with M/m-BACOD regimens.[10]

ciated with response or survival, as was increased LDH level and anemia.[7,9,23] There was some discrepancy concerning the value of morphologic subtype as prognostic indicator,[5,7,9,10,23,24] and most studies did not include patients with high-grade ML. With newer regimens additional prognostic indicators were recognized, such as large tumoral mass (abdominal or not) and the number of extranodal sites of disease.[10] A major difference compared with the results obtained by the M/m-BACOD regimens was that bulky tumor was of little consequence in our series. Only the presence of two or more large tumor masses was associated with poor prognosis and disappeared in multivariate analyses. In contrast with earlier studies that suggested an adverse association between prognosis and specific sites of disease[5,9] such as gastrointestional (GI) tract, spleen, or CNS, our results and those of Shipp et al[10] suggest that it is not the location of the disease that is significant but rather the number of sites.

Although the score described by Shipp was found to have some prognostic significance, the tumoral mass and the number of extranodal sites of disease were not the most significant prognostic factors in our study. While our patients had more indices of poor prognosis, their predicted 5-year survival rates were higher than Shipp's, with a small difference for the best prognostic group (77% *v* 68%), but a large difference for the poor risk patients (52% *v* 24%). This would appear to reflect the better results with our regimen than with the M/m-BACOD, but the actual difference, if any, could only be proved by a randomized study.

Drug intensity during the induction phase[14] was of borderline significance for predicting CR duration. It seems to us that drug intensity in our regimen is one of the factors responsible for the better results obtained, but it is difficult to demonstrate due to its low variation in our patients: those who received 75% of the theoretical dose received more adriamycin or cyclophosphamide per month than in CHOP or M/m-BACOD regimens.

The prognostic significance of the immunologic phenotype had rarely been addressed, probably because peripheral T cell lymphomas were rarely recognized in the past. Whether peripheral T lymphomas have the worst prognosis has been much debated and no firm conclusions can been drawn from the literature. Immunological phenotype is not correlated with other prognostic factors. The poor prognosis of our peripheral T cell lymphoma patients was essentially due to the large number of patients who died in induction, failed to respond, and relapsed during the first months of CR: median survival was only 14 months. The intensive regimen did, nevertheless, yield the best survival rates for peripheral T cell lymphomas compared with other forms of treatment.[25]

The LNH-80 regimen applied to 100 patients with aggressive ML can be credited with keeping 63 of them alive with a median follow-up of 4½ years. These results are the best published so far for this disease. Our results are currently being tested in a multicenter randomized study of the LNH-84 protocol in France and Belgium. More than 750 patients have already been included and the initial results will soon be communicated.

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Minimally Differentiated Acute Nonlymphocytic Leukemia: A Distinct Entity

By Edward J. Lee, Avrom Pollak, Richard D. Leavitt, Joseph R. Testa, and Charles A. Schiffer

Ten of 136 consecutive adult patients with previously untreated acute leukemia had morphologically undifferentiated leukemia by light microscopy. Leukemic cells from these patients were characterized by agranular cytoplasm, negative histochemical staining with sudan black (SB) and nonspecific esterase, and absent lymphoid cell surface markers and therefore were not classifiable according to the French-American-British (FAB) system. Electron microscopy with myeloperoxidase (MPO) staining revealed the presence of peroxidase positive cytoplasmic granules and endoplasmic reticulum in eight of the nine patients studied. Cells from the patient who was negative for MPO were also negative for platelet peroxidase. A series of monoclonal antibodies to myeloid antigens also revealed myeloid features with all patients having at least one myeloid differentiation antigen present on the surface of their cells. Common acute lymphoblastic leukemia (ALL) antigen was absent in the nine patients tested. Cytogenetic analysis of blast cells was abnormal in seven patients on whom adequately banded chromosomes were obtained although there were no consistent abnormalities. No patient had a Ph^1 chromosome. Only two of the ten patients achieved a complete remission. Morphologically undifferentiated leukemia may have myeloid features when studied by transmission electron microscopy or with monoclonal antibodies for cell surface markers. Such studies should be performed when the leukemia cannot be classified using either light microscopy or lymphoid cell surface markers. Such patients infrequently achieve remission with standard therapy and constitute a distinct entity.
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THE DISTINCTION between acute lymphoblastic leukemia (ALL) and acute nonlymphocytic leukemia (ANLL) can usually be made on the basis of light microscopy, polychrome stains, and cytochemical stains such as myeloperoxidase (MPO), sudan black (SB), and periodic acid Schiff (PAS). This distinction is important because of the differences between ALL and ANLL in the choice of chemotherapeutic agents, length of therapy, and propensity of ALL to involve the CNS. The biological basis for this distinction is the presumed origin of the leukemia from the malignant transformation of a stem cell that has undergone limited differentiation. The French-American-British Cooperative Group (FAB) has published standardized critieria by which ALL can be distinguished from ANLL and then further classified into subtypes.[1,2] ALL is defined as a leukemia in which the majority of cells have scant amounts of agranular cytoplasm, predominantly round nuclei, and homogeneous, slightly condensed chromatin without prominent nucleoli (L1 subtype). The leukemic cells can also be large, with abundant vacuolated basophilic cytoplasm, and prominent nucleoli (L3 subtype). Both of these variants can be readily distinguished from ANLL in Romanowsky polychrome stained preparations. A third subtype of ALL is characterized by large cells that exhibit some heterogeneity in size and have irregular nuclear shape with one or more large nucleoli (L2 subtype). Although blasts from patients with ANLL often contain cytoplasmic granules and thus can be easily distinguished from ALL, some have only small numbers of granules. Such patients with ANLL can then be best distinguished from L2 by cytochemical staining. Using arbitrary criteria, the FAB group states that the presence of <3% MPO positive cells suggests the diagnosis of ALL, whereas, if ≥3% of cells are positive, the diagnosis is ANLL.

In this report we present the results of studies of leukemic cells of ten patients with acute leukemia who did not meet these FAB criteria for ANLL. These patients had leukemic cells that were small, with scant agranular cytoplasm and distinct nucleoli. Auer rods were not seen and cytochemical stains (PAS, SB, chloroacetate esterase, α-Naphthyl butyrate esterase) were negative. The leukemic cells from these patients were studied by ultrastructure and for cell surface markers using monoclonal antibodies. Our data suggest that such undifferentiated leukemias frequently exhibit features that suggest a myeloid origin. Furthermore, our results suggest that these patients infrequently achieve remission when treated with conventional therapy for ANLL.

MATERIALS AND METHODS

Leukemic cells obtained from peripheral blood or bone marrow from 136 consecutive patients (Table 1) referred to the University of Maryland Cancer Center with previously untreated acute leukemia were analyzed. Thirteen patients (eight ALL, five ANLL) had inaspirable marrows at the time of diagnosis, and laboratory studies were performed on peripheral blood or cells obtained from a bone marrow biopsy core. Whenever possible, all of the following series of studies were performed.

Light microscopy. Bone marrow smears (or touch preparations from biopsy cores) and peripheral blood smears were stained with Wright-Giemsa for cell morphology and differential, with histochemical stains including SB, PAS, chloroacetate esterase, and alpha-naphthyl butyrate esterase. Terminal deoxynucleotidyl transferase (Tdt) was detected by indirect immunofluorescence (Bethesda Research Laboratory Inc, Gaithersburg, MD) using the method of Bollum.[3]

Immunologic cell surface marker studies. Cell surface marker studies using a variety of monoclonal antibodies and indirect immunofluorescence microscopy were done using standard methods described elsewhere.[4] Monoclonal antibodies were used to identify

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Table 1. Patient Characteristics

	Total	de novo ANLL	2^0 ANLL	ALL	M-O
N (no. evaluable for response to therapy)	136 (129)	94 (90)	16 (14)	16 (15)	10 (10)
Median age (yr)	60	61	60	58	52
(range)	(15-83)	(15-81)	(29-77)	(20-83)	(34-71)
Male	69	48	7	10	5
Female	65	46	9	6	5
Complete response (% of evaluable patients)	53	63	36	53	20

Abbreviations: de novo ANLL, no previous hematologic disorder; 2^0 ANLL, chemotherapy, radiotherapy, or another stem cell disorder preceded the diagnosis of leukemia; M-O, minimally differentiated ANLL.

differentiation antigens of T lymphocytes, B lymphocytes, monocytes and granulocytes, as well as to identify the presence and nature of surface or cytoplasmic immunoglobulins. An antigen was considered present if >20% of cells studied were positive.

Ultrastructural studies. Bone marrow particles were fixed in a phosphate-buffered formaldehyde-glutaraldehyde mixture and further processed for transmission electron microscopy (TEM) in a routine fashion. For the ultrastructural localization of platelet-specific peroxidase, the tissue was first fixed in a tannic acid-aldehyde mixture.[5] Thereafter, the material for peroxidase staining was incubated in 3,3′-diaminobenzidine (DAB) medium, fixed, embedded, and sectioned.

Cytogenetic analyses. Procedures for the cytogenetic analysis of leukemia patients have been described in detail elsewhere.[6] Briefly, marrow cells were examined from 24 hour unsynchronized cultures and methotrexate-synchronized cultures. Chromosomes were examined with conventional Giemsa stain and quinacrine fluorescence banding techniques. All chromosome studies were performed at diagnosis, before initiating therapy. Chromosomes were identified and karyotypes assigned according to the International System for Human Cytogenetic Nomenclature (1985).[7]

Patients were classified by light microscopy according to the criteria of the FAB schema for both ANLL and ALL.[1,2]

RESULTS

Of the 136 patients analyzed, 110 (81%) had ANLL, 16 (12%) had ALL, and ten patients (7%) had leukemia that was not classifiable by conventional morphologic and cytochemical criteria (Table 1). There were no clinical characteristics, abnormalities on physical examination or initial laboratory parameters that distinguished these ten patients. One of these patients (no. 8) developed leukemia after extensive chemotherapy and radiotherapy for Hodgkin's Disease. No history of mutagen exposure was noted in any of the other nine patients. A second patient (no. 6) had a low WBC count for 4 to 5 months before developing leukemia but no other patient had a hematologic disease before the diagnosis of acute leukemia. The results of laboratory investigations on these ten patients are summarized in Tables 2 and 3.

Light microscopy. Marrows were hypercellular in all but one patient. Six patients had only occasional cells identifiable that were not leukemic whereas the other four patients had easily identified populations of residual mature myeloid or erythroid cells. In all of these patients, the leukemic cells were small with sparse, agranular, lightly basophilic cytoplasm, round nuclei, immature chromatin and prominent nucleoli (Fig 1). Auer rods were absent. In all

Table 2. Hematologic and Cytogenetic Features

Patient No.	Peripheral Blood				Bone Marrow		Karyotype
	WBC*	BL (%)	HCT (%)	PLT*	Cellularity†	Blasts (%)	
1	3.0	0	33	56	3	50	Banding inadequate
2	175	100	36	80	4	99	48,XX,+X,+8,t(1;19)(p34;q13)
3	8.0	27	27	84	3-4	95	45,XY,−5,−7,−10,−11,−17,+r,+3mar/46,XY
4	51	94	22	51	3	99	46,XX,dic 17(p11)
5	1.8	0	36	99	3	80	45,XX,−3,−14,−20,del(2)(p2?1),del(7)(p1?5),del(12)(q15q24),+der(3)t(3;?)(q2?8;?),+der(20)t(14;20)(q24;p13)
6	2.6	20	27	21	3	50	46,XY,−7,+r/46,XY
7	98	100	22	34	3	99	45,XY,−1,−13,−15,del(13)(q12q14),t(8;14)(q24;q32),+der(1)t(1;15)(p36;q1?3),+der(13)t(13;?)(q34;?)/46,XY,−1,−6,−13,−15,del13,t(8;14),+der(1)t(1;15),+der(6)t(6;?)(p25;?),+der(13)t(13;?),+mar
8	6.5	28	26	30	2-3	60	46,XX,r18(p11q21)/46,XX
9	0.6	0	38	210	2	95	Banding inadequate
10	0.6	5	23	20	2-3	95	46,XY

Abbreviations: WBC, white blood count; BL, blast percentage; HCT, hematocrit; PLT, platelet count.

* × $10^3/\mu L$.

†Graded as 0-4/4.

Table 3. Cell Surface Marker Analysis and Electron Microscopy

	TdT	HLA-DR*	MCS-1†	MCS-2†	MY-10‡	MY-11‡	MY-26‡	OKM-1§	CALLA‖	OKT-3§	OKT-6§	OKT-10§	B1¶	SmIg	CyIG	MPO#
Reference	3		8	8	9		10	11	12	13	13	14		4	4	
CD			CDw15	CDw13				CD11	CD10	CD3	CD1		CD20			
Patient no.																
1	20%	69%	5	10	52	69	ND	14	1	5	ND	ND	ND	ND	ND	Pos
2	Neg	83	0	2	78	78	71	81	1	2	ND	ND	ND	1	ND	Neg
3	40%	38	ND	42	41	77	ND	42	2	5	2	10	4	ND	ND	Pos
4	Neg	85	ND	13	74	11	ND	16	2	7	0	30	16	0	0	Pos
5	Neg	ND	ND	3	53	13	ND	9	2	12	63	ND	ND	ND	ND	Pos
6	Neg	30	25	30	ND	ND	ND	ND	2	ND	ND	ND	ND	ND	ND	Pos
7	Neg	89	0	3	3	94	1	2	3	3	ND	ND	ND	ND	ND	Pos
8	Neg	48	6	52	21	26	21	28	2	10	ND	ND	ND	ND	ND	ND
9	Neg	ND	4	26	ND	ND	ND	ND	5	ND	ND	ND	ND	ND	ND	Pos
10	Neg	46	0	47	60	67	5	0	3	8	ND	ND	ND	ND	ND	Pos

All numbers represent percent of cells positive.

Abbreviations: CD, cluster designation (as indicated by the first and second International Workshops on Leucocyte Differentiation Antigens); ND, not done; Pos, positive; Neg, negative.

*From Becton Dickinson, San Francisco.

†Gift of Dr Jan Minowada.

‡Gift of Dr Curt Civin.

§From Ortho Pharmaceuticals, Raritan, NJ.

‖Gift of Dr Jerome Ritz.

¶From Coulter Immunology, Hialeah, FL.

#Myeloperoxidase stain by transmission electron microscopy.

patients, <3% of the blasts were positive with cytochemical staining for SB and staining with specific or nonspecific esterases and PAS was uniformly negative. Thus, although the appearance of the leukemic cells did not suggest lymphoid origin, cytochemical criteria for the suspected diagnosis of ANLL were not met.

Leukemic cells were evaluated for the presence of Tdt by indirect immunofluorescence in all ten patients. In one patient (no. 1), 20% of the blasts were positive and in a second (no. 3), 40% were positive. The other eight patients each had <5% of their leukemic cells stain positively.

Ultrastructural studies. TEM was performed on leukemic cells of nine of the ten patients. Blast cells in all patients appeared extremely undifferentiated on TEM. The nuclei contained primarily euchromatin with little heterochromatin, and large nucleoli were seen. With MPO staining (Fig 2), eight of the nine patients had leukemic cells containing small numbers of MPO positive granules in the cytoplasm in 6% to 40% of the blasts. Other cells had MPO-positive material in endoplasmic reticulum and nuclear membrane. MPO-positive cytoplasmic granules are not found in megakaryocytes or their precursors, or in cells of lymphoid lineage. Except when cells are fixed with tannic acid, the presence of MPO staining in endoplasmic reticulum and nuclear membrane identifies cells of myeloid lineage.[5] Thus, these patients were thought to have leukemia derived from a very early precursor cell committed to myeloid differentiation. Only patient no. 2 had leukemic cells lacking MPO activity, and megakaryocyte-specific peroxidase was also absent from bone marrow and peripheral blood cells examined after tannic acid fixation. Thus, eight of nine patients studied with TEM had evidence suggesting myeloid origin of their leukemic cells.

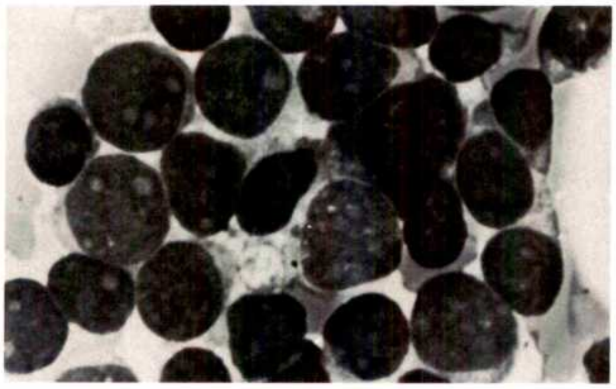

Fig 1. Photomicrograph of Wright-Giemsa stained bone marrow smear from patient no. 6. Several blasts contain scant cytoplasm and prominent, often multiple nucleoli. No Auer rods or cytoplasmic granulation can be identified. (Original magnification ×1,000; current magnification ×400.)

Cell surface markers. Leukemic cells from all ten patients were evaluated for the presence of cell surface antigens with monoclonal antibodies to normal myeloid differentiation antigens.

Myeloid differentiation antigens were present on the leukemic cells of all 10 patients. All but two patients had several such markers identified on their cells (Table 3). No consistent pattern of reactivity was seen although My-10 (seven of eight patients) and My-11 (six of eight) were frequently found.

The common ALL antigen was not detected on the cells in any of the patients. T-cell differentiation antigens were found infrequently. The cells of two patients (no. 4 and 5) had a single such antigen. Leukemic cells from three patients were evaluated for B lymphocyte markers. None of these studies were positive, whereas all specimens tested were positive by HLA-DR (Ia-antigen).

Chromosomal analyses. Cytogenetic analyses were per-

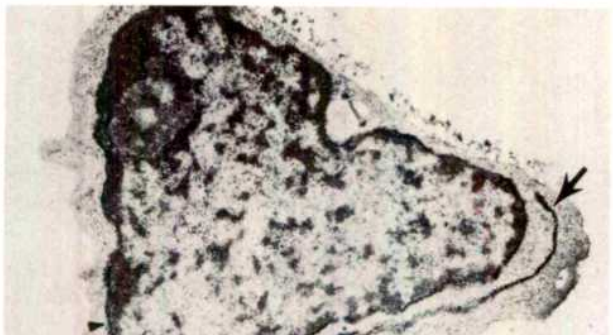

Fig 2. (A) Electron micrograph of a blast cell from patient no. 4 after formaldehyde-glutaraldehyde fixation and MPO staining. The nucleus contains a large nucleolus and little heterochromatin. The nuclear membrane stains positively with MPO (arrowheads). The cytoplasm contains endoplasmic reticulum, which also stains positively for MPO (arrows). (Original magnification ×14,000; current magnification ×11,060.) (B) Electron micrograph of a blast cell from patient no. 6 after formaldehyde-glutaraldehyde fixation and MPO staining. The nucleus contains a large nucleolus and little heterochromatin. The cytoplasm contains mitochondria, endoplasmic reticulum, and MPO positive granules (arrows). (Original magnification ×9,300; current magnification ×7,350.)

formed in all ten patients. No patient had a Ph^1 chromosome. Two patients (no. 1 and 9) had poorly banded chromosomes that precluded detailed analysis. Seven of the eight patients who had adequate metaphases examined had an abnormal karyotype. Two patients (no. 3 and 6) had loss of one no. 7 chromosome, and a third (no. 5) had loss of part of the short arm of one no. 7 chromosome. One no. 17 chromosome was missing in one patient (no. 3), and a second patient (no. 4) had a dic(17) that resulted in loss of part of the short arm of the no. 17 chromosome. Two patients (no. 2 and 7) had translocations involving one no. 1 chromosome but with the involvement of different chromosomes. One of the latter patients (no. 7) also had t(8;14). No other chromosome was involved in more than a single patient. Patient no. 10 had a normal karyotype.

Response to therapy. The ten patients with FAB-unclassifiable acute leukemia all received intensive chemotherapy. Eight patients were treated with daunorubicin (DNR) and cytosine arabinoside (Ara-C) according to the protocol therapy described below for ANLL. Two of these patients also were treated with vincristine and prednisone, one (no. 3) because of the presence of Tdt, and the other (no. 2) because of the absence of platelet or MPO staining on electron microscopy. One of these eight patients achieved a complete remission (CR). Two patients (no. 1 and 4) were treated with a regimen developed for ALL consisting of tandem methotrexate and L-asparaginase in combination with vincristine and dexamethasone.[15] One of these two patients, also with a low number of TdT-positive cells, achieved a CR. Of the eight patients in whom CR was not achieved, one (no. 7) died on day 15 with an aplastic bone marrow, while all seven of the remaining patients had refractory leukemia.

Of the 110 patients with ANLL diagnosed by routine criteria, 104 were evaluable for response to chemotherapy. Five patients who were not treated and one who was lost to follow-up were inevaluable. Of these 104 patients, 57 of 90 (63%) with de novo ANLL achieved CR (Table 1). Five of 14 (36%) patients with secondary ANLL achieved CR. Treatment of these 104 patients consisted of protocol chemotherapy (99 patients) with DNR 45 mg/m^2 daily for three days, and Ara-C by continuous infusion for seven days at a dosage of either 100 mg/m^2 before December 1982[16] or 200 mg/m^2 after January 1983.[17] Five patients who received other regimens did not achieve a complete response.

Of the 16 patients with ALL, eight of 15 achieved CR after treatment with either DNR, vincristine, prednisone, and L-asparaginase[18] or with a combination of methotrexate and L-asparaginase given in a tandem fashion with vincristine and dexamethasone.[16] One patient refused treatment.

DISCUSSION

The ten patients described here did not meet the FAB criteria for any of the various subtypes of acute leukemia. The diagnosis of ANLL could be made only by ultrastructural MPO staining or cell surface marker studies. Eight of the patients (no. 1, 3, 4, 5, 6, 7, 9, and 10) had MPO positive cells and, on this basis, had ANLL. The FAB group suggests that the demonstration of MPO positive blast cells by TEM is sufficient to establish myeloid lineage.[2] The number of cells with MPO positive material varied considerably, from 6 to 40%, in different patients. It is likely that this underestimates the frequency of positive cells because the thin sections necessary for TEM may by chance not detect the presence of small granules. With MPO at the light microscopy level, an arbitrary minimum percentage of 3% has been set by the FAB group; no such "definition" exists for ultrastructural MPO. The variation in both the percentage of positive cells and the degree of "positivity" per cell emphasizes the considerable heterogeneity of morphologic differentiation occurring even within the same patient.

In the other two patients, the diagnosis of ANLL depended solely on cell surface marker studies. All ten patients tested positively for myeloid antigens, and in two patients (no. 2 and 8), this was the only evidence of lineage specificity. When MPO or platelet PO activity is absent (patient no. 2) or cannot be established (patient no. 8), the constellation of four different positive "myeloid" monoclonal antibodies as for patient no. 2, and five different positive "myeloid" monoclonal antibodies, as for patient no. 8, is sufficient to indicate myeloid features and to confirm the diagnosis of ANLL. This is substantiated by the presence of OKM-1 in both patients no. 2 and 8. A recent review notes that OKM-1 has not been reported in cells from any of 92 patients with lymphoid leukemias.[19] Other monoclonals positive on cells from these two patients are less lineage-specific. My-10 and My-11 have been identified on both ALL and ANLL.[9] The absence of Tdt and CALLA in these two patients in conjunction with several positive myeloid antigens confirms the presence of a very early cell committed to myeloid differentiation.

There have been a number of descriptions of patients with characteristics suggestive of both ALL and ANLL, examples of so-called "lineage infidelity" or biophenotypic leukemia.[20-22] Four of our patients may fit into this category. Two patients (no. 1 and 3) had Tdt detected in a minority of their blasts. Tdt positivity was detected in 19 of the 110 (17%) patients with classifiable ANLL in our study. Some may have been due to nonspecific Fc receptor mediated binding of reagents in that they were less positive after incubation of cells in AB plasma. Others, however, seemed to represent "true" positives. The presence of Tdt in some patients with ANLL has been confirmed by other investigators using a biochemical method[21] of identifying Tdt activity, and has recently been identified in as many as 22% of patients with ANLL using an immunoperoxidase method.[23] Tdt has also been detected in immature cells in regenerating bone marrow[24] and in a recent review by Ben-Bassat and Gale,[25] it was stated that Tdt positivity alone in association with myeloid characteristics is insufficient to confirm the suspicion of biophenotypic leukemia.

Similarly, antibodies raised against thymic cells (OKT-6 and OKT-10), were reactive in two MPO positive, TdT negative patients (no. 4 and 5). Reinherz et al[26] found that the spectrum of OKT-10 positive cells included up to 20% of cells in normal bone marrow, suggesting that the determinant recognized by this antibody is not restricted to lymphocytes, and represents an "early differentiation antigen." Similarly, OKT-6 has been reported to be reactive with malignant cells from patients with histiocytosis X,[27] and with the myeloid cell line HL-60 after stimulation with gamma interferon.[28] Neither patient was studied for the presence of T-cell receptor gene rearrangements.

The diagnosis of acute megakaryoblastic leukemia (FAB-M7) was considered and excluded in nine of the ten patients. In immature megakaryoctyes, the peroxidase stain is positive only after fixation in tannic acid, and peroxidase-positive material is present in the nuclear membrane and endoplasmic reticulum.[5] Two patients with the M7 subtype were identified within the group of 110 with ANLL using this technique. One of the ten patients studied had neither MPO nor megakaryocyte peroxidase positive cells in either peripheral blood or bone marrow. Eight patients had MPO positivity on TEM with standard fixation, a finding not found with M7 leukemia. The FAB-M7 subtype was therefore definitively excluded in these nine patients.

These patients thus had leukemia that lacked features that are currently recognized as diagnostic of ALL or ANLL within the FAB schema. The FAB group recognizes that unclassifiable leukemias occur, but estimates the frequency at 2% in adults.[2] They describe this group as a mixture of patients with basophilic leukemia, hypoplastic ANLL, and ANLL requiring ultrastructural peroxidase studies "to confirm their granulocytic nature." In our series, ten of 136 patients (7%) fit this last category. In a series of patients reported by Youness et al,[29] 22 of 225 patients (10%) were thought to have acute undifferentiated leukemia (AUL), defined by the investigators as patients in whom light microscopy with the usual battery of cytochemical stains was not helpful in classification in the FAB schema. As in our patients, the leukemic cells were described as undifferentiated agranular cells with more cytoplasm than an L1 blast, round nuclei and one to two nucleoli. Of these 22 patients, 14 were classified as ANLL on the basis of TEM and MPO staining. In a third series, four of 56 (7%) ANLL patients were classified as AUL, but were defined only as "unclassifiable."[30] A fourth series described nine of 202 (4%) with either AUL or "biphenotypic" leukemia with leukemic cells similar to those described in our patients.[31] Despite the lack of uniform definition, all these series identify patients who had undifferentiated leukemia that was nonetheless considered myeloid by varying criteria. This subtype occurs with a frequency similar to that of the M3, M6, or M7 subtypes suggesting that the FAB group has underestimated the frequency of the entity of minimally differentiated acute myeloid leukemia.

Some patients with minimally differentiated ANLL may have been classified as ALL in many centers. A recent report from Cancer and Leukemia Group B (CALGB) of 129 adult patients treated for ALL identified four patients (3%) who lacked both B cell and T cell markers.[22] These patients exhibited only myeloid differentiation antigens when studied with monoclonal antibodies. A French group reported 25 adult patients with "ALL" who lacked both B cell and T cell markers.[32] Five patients studied by TEM with peroxidase staining demonstrated myeloid blasts.

No consistent cytogenetic abnormality was identified in the group of patients with minimally differentiated ANLL, but the number of patients is small. Seven patients with adequate mitoses for detailed analysis had structural rearrangements. One of these alterations (patient no 7), t(8;14) (q24;q32), is typically found in Burkitt's lymphoma and ALL-L3. Surface immunoglobulin was not evaluated in cells from this patient, although ultrastructural MPO was positive. Many of the other chromosome changes (eg, +7,+8, dic(17), and ring chromosomes) have been reported both in ANLL and ALL. Four of the seven patients had complex karyotypes similar to those in ANLL patients described by Sakurai and Sandberg[33] as "major karyotypic abnormalities" (MAKA). Among the remaining three patients, two had ring chromosomes, also a common finding in patients with MAKA. Although one patient (no. 8) with a ring chromosome had a history of prior therapy for Hodgkin's disease and was thought to have a therapy related acute leukemia, the other patients had no history of prior hematologic disease, malignancies, or mutagen exposure. In reviewing the previous mentioned series, only one (Youness et al)[29] examined karyotypes, and the vast majority of cases were examined without the benefit of banding techniques. In the three patients studied with banding, one had a MAKA, a second had two abnormal clones identified, both with different abnormalities of chromosome no. 13, and the third had an abnormality of chromosome no. 21.

Last, the response rate in these ten patients was extremely poor. Treatment in eight patients with standard ANLL chemotherapy produced one brief CR lasting 5 months and six patients had resistant leukemia. The other patient who achieved CR, the eldest of the group, was treated with a regimen active in both ALL and ANLL, and died of complications of further chemotherapy 4 months after achieving CR. These results differ significantly from the 60% CR in the 104 concurrently treated patients with ANLL who were classifiable according to the FAB system ($P = .02$, Fisher exact test). Youness et al found a CR rate of 68% in the 22 patients reported, but the median survival time was only 26.7 weeks.[29] This is considerably shorter than the median survival for patients with ANLL treated at the same institution.[34] In the four patients described by Foon et al,[30] three achieved complete response but data on survival are not available. Mertlesman et al[31] noted both a decrease in the remission rate and shortened duration of survival and remission in their mixed group classified as AUL although statistical significance is not reached due to small patient numbers. In reviewing their results in patients with minimally differentiated ANLL treated as ALL, Reiffers et al noted that all five patients who were MPO-positive by TEM were among the group of seven patients in whom treatment failed to produce a remission.[32] Lastly, CALGB noted that none of the four patients with what they termed "myeloid" ALL achieved CR when treated with daunorubicin, vincristine, prednisone, and L-asparaginase.[22]

The ten patients described here have leukemia that is nonlymphocytic by morphology, ultrastructure, and cell surface markers. The ability to prospectively identify a group of patients with poor response to chemotherapy suggests that minimally differentiated ANLL is indeed a clinically distinct subtype of acute leukemia. This leukemia is presumably derived from very early committed granulocyte precursors. Our series and those previously cited suggest that an M-O category for ANLL be created and defined by the presence of MPO detectable only on the ultrastructural level. The absence of lymphoid markers and the presence of myeloid differentiation antigens is confirmatory, and identify patients in whom TEM with MPO should be examined. It would appear that the prognosis for patients with minimally differentiated ANLL is poor using standard therapeutic approaches.

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Evidence for the Interleukin-2 Dependent Expansion of Leukemic Cells in Adult T Cell Leukemia

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Interleukin 2 (IL-2) receptor/Tac antigen is abnormally expressed on cells of patients with adult T cell leukemia (ATL) caused by infection with human T lymphotropic virus type I (HTLV-I). Twenty-five patients with ATL were examined to determine whether their leukemic cells continued to show IL-2–dependent proliferation. In 21 patients, the in vitro proliferation of HTLV-I-infected nonleukemic T cell clones was found to be dependent on IL-2. However, clonality analysis based on T cell receptor gene rearrangement profiles and the site of HTLV-I provirus integration revealed IL-2–dependent growth in leukemic cells in four patients with ATL. These results provide evidence for the IL-2–dependent proliferation of leukemic cells in some ATL patients.

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ADULT T CELL LEUKEMIA (ATL),[1-4] endemic in southwest Japan, the Caribbean Islands, and Africa, is etiologically associated with human T lymphotropic virus type I (HTLV-I).[5-7] Leukemic cells in ATL consist of monoclonal HTLV-I infected T cells[8] with the surface phenotype of helper T cells,[2,9] and they continuously express an excess number of IL-2 receptors.[10-12] Such constitutive IL-2 receptor production by both HTLV-I and HTLV-II–infected helper T cells has been shown to be induced by the *pX* or *tat* genes of the two viruses.[13,14] In contrast, normal mature T cells transiently express IL-2 receptors when stimulated by antigens or mitogens, resulting in transient IL-2–induced cell proliferation.[15] Since T cell proliferation is regulated by the interaction between IL-2 and IL-2 receptors, the abnormal expression of IL-2 receptors on ATL leukemic cells is thought to be responsible for their unregulated growth.[16] Indeed, using IL-2 as a growth factor, long-term cultures of T cells were established from ATL patients,[17-19] but rarely from HTLV-I noninfected healthy donors. However, analyses of the rearrangement profiles of the T cell receptor gene revealed that most of these ATL-derived T cell lines did not represent major clones of the original ATL leukemic cells.[20] Peripheral leukemic cells from ATL patients did not grow in the presence of IL-2.[20,21] However, we found a chronic ATL patient whose leukemic T cells were able to grow as a permanent cell line in the presence of IL-2. This cell line's rearranged T cell receptor gene profile was identical to that of the patient's peripheral leukemic T cells.[20] We therefore speculated that IL-2 acts as a growth factor for HTLV-I–infected T cells in some stage of ATL development.

We extended our work to search for similar cases and, here we report four patients whose leukemic T cells proliferated in the presence of IL-2.

MATERIALS AND METHODS

Patients. Four male patients were diagnosed as having ATL on the basis of clinical and hematological findings. ATL was further confirmed by the presence of a monoclonal population of HTLV-I–infected T cells, identified by Southern blot hybridization using as a probe cDNA of the U3R portion of the HTLV-I genome. The peripheral blood leukocyte (PBL) count and percentage of atypical lymphocytes were 46,000/μL (67%) in patient no. 1 (55 years), 15,500/μL (55%) in patient no. 2 (66 years), 30,900/μL (47%) in patient no. 3 (42 years), 37,500/μL (53%) in patient no. 4 (35 years). Patients no. 1 and 2 had acute ATL, and patients no. 3 and 4 had chronic ATL.

Cells and cell culture. Mononuclear cells were separated by Ficoll-Hypaque centrifugation (LSM solution, Litton Bionetics, Kensington, MD) from heparinized samples of the patients' peripheral blood. Cells were cultured at an initial cell density of 5 to 10 × 10^5 cells/mL in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 100 μg/mL kanamycin and 10% to 20% fetal bovine serum (FBS) (Rehatuin, Armour Pharmaceutical Co, Kankakee, IL) in the presence of crude or recombinant IL-2. Cells were subcultured every three to five days by spliting them at a two to five fold dilution depending on their density. Cells were cultured in 55-mm petri dishes (Falcon) at 37°C in a humidified atmosphere of 5% CO_2 in air.

IL-2 preparation. Conditioned medium containing IL-2 was prepared by culturing mitogen-stimulated peripheral blood lymphocytes and spleen cells as described.[20] Recombinant IL-2 produced in *Escherichia coli* was kindly supplied by Dr Y. Sugino (Takeda Chemical Industries, Osaka, Japan). For T cell growth stimulation crude IL-2 was used at a 25% to 50% (vol/vol) concentration in RPMI 1640 medium, and purified recombinant IL-2 was used at 1 to 5 U/mL (1 U/mL = 7.5 ng/mL).

Southern blot hybridization. High molecular weight DNA was extracted from peripheral blood mononuclear cells of ATL patients and from IL-2–dependent T cell line cells derived therefrom. Procedures for DNA extraction, digestion by restriction enzymes, electrophoresis, blotting to nitrocellulose filter, and hybridization with ^{32}P-labeled molecular probes are described elsewhere.[22] To analyze T cell receptor gene rearrangement, a 2.6 kilobase (kb) *Hind*III fragment, containing the constant region of the human β chain, was

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used as the probe.[23] To detect HTLV-I provirus the long terminal repeat (LTR) (*Sma*I-*Sac*I, 456 base pairs [bp]) was used as the probe.[24]

Examination of the cell surface phenotype by monoclonal antibody. The surface phenotypes of fresh and cultured ATL cells were examined by indirect immunofluorescence analysis using OK-series (Becton Dickinson, Oxnard, CA), Leu-series (Ortho Diagnostic Systems, Raritan, NJ) and anti-Tac monoclonal antibodies. Fluorescein-isothiocyanate (FITC)–conjugated goat anti-mouse immunoglobulin (Cappel) was used as the second antibody. The results were expressed based on cluster and differentiation (CD) classification.[25] Fluorescence intensity and the percentage of fluorescence positive cells was examined by an Ortho Spectrum III (Ortho Diagnosis Systems, Sunnyvale, CA) flow cytometer.

RESULTS

IL-2–dependent growth of ATL leukemic T cells. Peripheral blood mononuclear cells from patients no. 1 through 4 were seeded in 10% FBS supplemented RPMI 1640 medium in the presence of either crude or recombinant IL-2. The cells began to grow without a lag phase, and after several weeks showed stable growth that was dependent on exogenous IL-2, though the growth pattern varied among cells from different patients. T cell lines derived from patients no. 1, 2, and 4 have been maintained as leukemic clones in in vitro culture for more than a year. Characterization of these T cell lines was carried out after 3 months in culture. A T cell line derived from patient no. 3 has been maintained as leukemic clones for more than 3 months and the Southern blot hybridization study was carried out after 2 weeks in culture. The growth curves for these four T cell lines are shown in Fig 1.

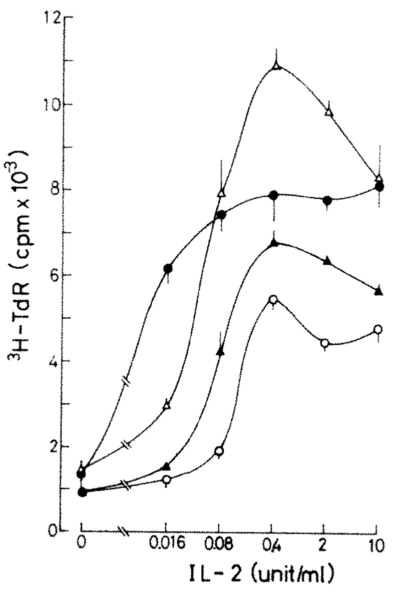

Fig 1. The effect of IL-2 on DNA synthesis in cultured ATL leukemic cells. Leukemic T cells in long-term culture derived from patient no. 1 (●—●), patient no. 2 (O—O), patient no. 3 (▲—▲), and patient no. 4 (△—△), were cultured in the presence of different concentration of purified recombinant IL-2 in 96-well microtiter plate and cultured for 72 hours or 120 hours (patient no. 2). Cells were seeded at 2 × 10^4/200 µL/well or 1 × 10^4/200 µL/well (patient no. 2). Cells were pulse labeled with methyl-^{3}H thymidine (2 Ci/mmol, Amersham) at 1 µCi/mL for the last four hours of culture, and were harvested on glass-fiber filter paper using a cell harvester (Bellco Co, Vineland, NJ). ^{3}H-TdR incorporation was counted by a Packard Mark II scintillation counter. Results were expressed as the mean ± SD based on triplicate assays.

Characterization of the ATL and cultured cells. As shown in Table 1, peripheral blood ATL cells of three patients were CD 4 (OKT-4/Leu-3a)-positive, whereas those of patient no. 3 were CD 4- and CD 8 (OKT-8/Leu-2a)-positive. All cultured cells were found to be T cells (CD 2^+), and showed the same surface phenotype as the fresh ATL cells. In addition, all the cultured cells overexpressed the IL-2 receptor/Tac antigen (CD 25^+, data not shown).

Clonality analysis of the cultured T cells. As every T cell clone expresses different T cell antigen receptor, identifiable by the T cell receptor gene rearrangement, T cell clonality can be analyzed by examining the T_β gene rearrangement. Such rearrangements are visualized by decreases in the intensities of the germ line bands (*Eco*RI, 4 kb and 10.5 kb/ *Bam*HI, 23 kb; as shown in Fig 2), and by the appearance of one or two new bands. T cells obtained from the four ATL patients were found by Southern blot hybridization to consist of monoclonal populations, each with a different T_β gene rearrangement (no. 1, 3, 5, and 7 in Fig 2A, a and b). The cultured T cells also contained monoclonal populations (no. 2, 4, 6, and 8 in Fig 2A, a and b). Moreover, the T_β gene rearrangement profiles of the fresh ATL cells were identical to those of their derived T cell lines (no. 1 and 2; no. 3 and 4; no. 5 and 6; no. 7 and 8 in Fig 2A, a and b).

HTLV-I genome in the ATL leukemic and cultured T cells. In ATL, HTLV-I provirus is usually integrated as a single copy into leukemic cells, and can be detected by Southern blot hybridization using a fragment of HTLV-I cDNA as a molecular probe. *Eco*RI digestion of all four ATL patients' peripheral blood mononuclear cell DNA revealed a single band (no. 1, 3, 5, and 7 in Fig 2B). HTLV-I provirus was also detected in the cultured T cell lines (no. 2, 4, 6, and 8), although multiple bands were detected in three cases. Moreover, when the original ATL cells and their derived T cell lines were combined, a common band was

Table 1. Surface Phenotypes of ATL Cells and Their Derived Cultured T Cells

Patient No.	PBL/Cultured T cells (T)	CD 5 (%)	CD 4 (%)	CD 8 (%)	CD 2 (%)	CD 25 (%)	OK-Ia/HLA-DR (%)
1	PBL	98.9	97.2	1.3	NT	80.4	71.5
	T	NT	99.6	0.1	99.7	99.3	98.5
2	PBL	79.2	95.6	1.5	NT	92.5	91.7
	T	NT	93.1	0.7	97.4	98.8	98.7
3	PBL	NT	70.7	60.7	87.0	88.1	NT
	T	NT	31.4	97.5	99.9	99.6	NT
4	PBL	NT	89.0	3.9	92.6	20.4	43.3
	T	NT	98.9	1.2	99.5	88.1	NT

Viable cells were treated with mouse monoclonal antibodies (Leu-series, OK-series and anti-Tac) followed by staining with FITC-conjugated anti-mouse IgG goat antibody F(ab') fragment. Stained cells were examined using Ortho Spectrum III flow cytometer. Surface phenotypes were expressed based on cluster and differentiation (CD) classification.

Abbreviation: NT, not tested.

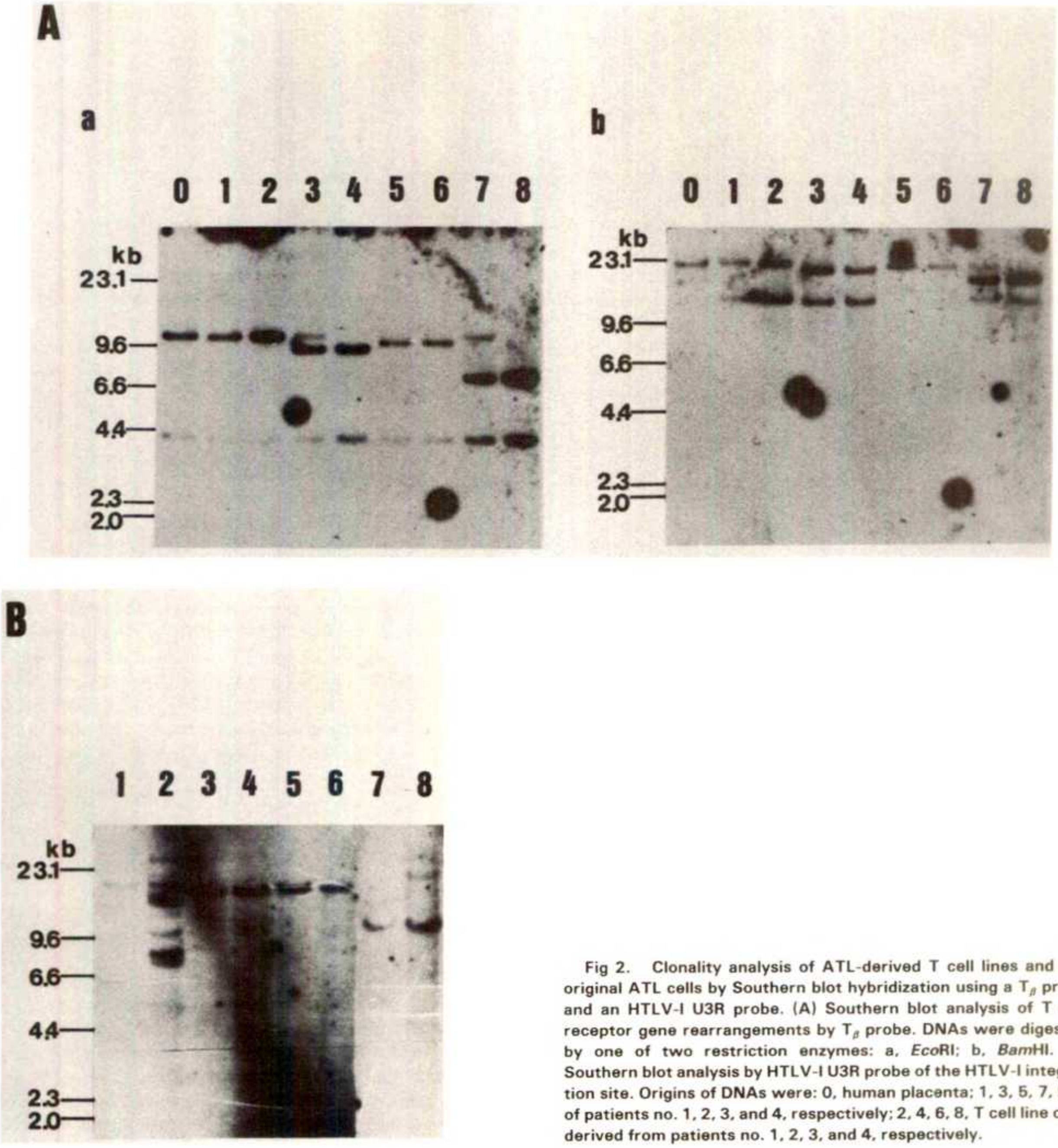

Fig 2. Clonality analysis of ATL-derived T cell lines and the original ATL cells by Southern blot hybridization using a T_β probe and an HTLV-I U3R probe. (A) Southern blot analysis of T cell receptor gene rearrangements by T_β probe. DNAs were digested by one of two restriction enzymes: a, *Eco*RI; b, *Bam*HI. (B) Southern blot analysis by HTLV-I U3R probe of the HTLV-I integration site. Origins of DNAs were: 0, human placenta; 1, 3, 5, 7, PBL of patients no. 1, 2, 3, and 4, respectively; 2, 4, 6, 8, T cell line cells derived from patients no. 1, 2, 3, and 4, respectively.

observed (no. 1 and 2; no. 3 and 4; no. 5 and 6; no. 7 and 8 in Fig 2B).

DISCUSSION

The discovery of T cell growth factor (TCGF), or IL-2, made selective long-term growth of mature T cells possible,[26] and some investigators speculated that malignant T cells with mature T cell phenotypes could be selectively grown using IL-2 as a growth factor.[17,18,20,27]

Since ATL is a leukemia of mature T cells with helper/inducer phenotype,[2,9] whose cells express IL-2 receptor constitutively,[10-12] we thought it possible that IL-2 would be an effective growth factor for them. Indeed IL-2–dependent helper T cell lines could be established from the peripheral blood of ATL patients, but clonality analyses based on the T cell receptor gene rearrangements and the site of HTLV-I provirus integration revealed that most of these T cell lines were clonally different from ATL cells. They are believed to have been derived from HTLV-I–infected nontransformed T cells.[20,28] From a patient with chronic ATL, however, we established an IL-2–dependent T cell line that had the same T cell receptor gene rearrangement and HTLV-I genome integration profiles as the original leukemia cells.[20] From this ATL patient, we sequentially established four IL-2–dependent leukemic T cell lines, including the newly established T cell line derived over 7 months from patient no. 4 described here. The findings of IL-2 responsive ATL cells in his peripheral blood suggested that ATL cells might at some time during the disease be IL-2–responsive. Therefore, we attempted to grow IL-2–dependent T cells from more than 25 ATL patients, and subjected the cells obtained to clonality analysis to determine whether they were derived directly from the original ATL cells. To date, we have identified four ATL patients from whom ATL cell clones could be grown as IL-2–dependent T cell lines.

Although it was reported that leukemic cells of most patients with ATL did not respond to IL-2,[21] despite constitutive IL-2 receptor expression, some cases of IL-2–responsive ATL including our studies have recently been reported.[29]

An important question arises as to whether these IL-2–responsive ATL cases have clinical features distinct from the majority of ATL cases in which leukemic cells do not respond to IL-2. Although our first IL-2–responsive ATL case had a chronic clinical course, only one of the other three cases had a chronic to subacute course. Further clinical studies are required to delineate this point.

Furthermore, it has been found that ATL-derived IL-2–dependent T cells become IL-2–independent during long-term culture.[17-19,30] We also isolated IL-2–independent leukemic T cell clones from the IL-2–dependent T cell lines in patient no. 4 (unpublished).

These results suggest the presence of an IL-2–dependent growth phase of leukemic cells in some ATL patients. Such a phase may be in the early stages of ATL, because leukemic cells in the majority of ATL cases do not respond to IL-2. To examine this possibility in vivo, a follow-up study over 3 years on the patient no. 4 is in progress. It is worth mentioning that freshly isolated ATL cells of patient no. 1 grew in an autocrine fashion.[29]

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Acute Nonlymphocytic Leukemia Following Etoposide and Cisplatin Combination Chemotherapy for Advanced Non–Small-Cell Carcinoma of the Lung

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Combination chemotherapy is frequently used in the therapy of advanced non-small-cell lung cancer (NSCLC), but late complications are rarely recognized because of the short survival of most patients. Of 119 patients with advanced NSCLC treated with cisplatin and other drugs, four patients developed acute nonlymphocytic leukemia (ANLL). All four patients received etoposide and cisplatin with or without vindesine. Leukemia was diagnosed at 13, 19, 28, and 35 months after start of treatment. Three patients had morphologic and/or cytogenetic features of acute leukemia with significant monoblastic involvement; the fourth patient had trilineage dysplasia and cytogenetic abnormalities more commonly associated with therapy-related leukemia. Detailed analysis of the subgroup who survived longer than 1 year (24 patients) suggests that high cumulative doses of etoposide are leukemogenic; the median etoposide dose was 6,795 mg/m^2 (first year only) in the four leukemic patients compared with 3,025 mg/m^2 in the 20 nonleukemic patients ($P < .01$). The rate of ANLL was 0.30 per person-year after the first year (95% confidence limits 0.11 to 0.90), with a cumultative risk of 15% ± 11% at 2 years, and 44% ± 24% at 2.5 years. We conclude that high doses of etoposide are potentially leukemogenic, and can induce a syndrome with features of acute monoblastic leukemia de novo that is distinct from other secondary leukemias.

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ACUTE NONLYMPHOCYTIC LEUKEMIA (ANLL) is a known late complication of chemotherapy and/or radiotherapy.[1-12] Most reports to date explicitly implicate alkylating agents as the leukemogenic factor,[3,4,7] and the incidence of ANLL is higher in those patients treated with both alkylating agents and radiotherapy.[1] Therapy-related ANLL (t-ANLL) may be morphologically[13] and cytogenetically[9-11] distinguishable from acute leukemia arising de novo. Initial reports of t-ANLL were primarily among long-term survivors after curative therapy for Hodgkin's disease and non-Hodgkin's lymphomas.[1,2] More recently, this late complication of cytotoxic therapy has been reported in patients with small-cell carcinoma of the lung[5,6] and other solid tumors.[3,4,8,9,11,12]

Combination chemotherapy (often containing alkylating agents) is frequently used in the management of patients with advanced non–small-cell lung cancer (NSCLC), but late complications of therapy are rarely recognized due to the short survival of most patients.[14] Many recent studies have used cisplatin-based regimens, often in combination with etoposide and/or vindesine[14-16] to exploit possible synergy.[17]

We recently suggested in a preliminary report that the combination of cisplatin, etoposide, and vindesine is highly leukemogenic, based on the development of ANLL in three of 24 lung cancer patients who survived 1 year or more.[18] After further follow-up of this cohort of 24 patients, we now report a fourth case of ANLL. Analysis of these patients provides circumstantial evidence that etoposide may be the leukemogenic agent, and that etoposide may induce a leukemia with the morphologic and cytogenetic features of acute monoblastic leukemia (AMoL), rather than those more commonly seen in t-ANLL due to alkylating agents.

MATERIALS AND METHODS

From February 1981 to September 1984, 119 patients with stage III NSCLC were treated with cisplatin-containing regimens at the University of Chicago and Michael Reese Medical Centers. Patients with metastatic disease were entered into a phase III trial comparing three cisplatin-based regimens (Table 1) with a fourth arm using cyclophosphamide, doxorubicin, methotrexate, and procarbazine (CAMP).[19] Patients with unresectable stage IIIM$_0$ disease were entered in a phase II trial of vindesine, etoposide, and cisplatin (VEP) using the same schedule described in Table 1. The patients who received CAMP have been excluded from the present analysis, since they received cyclophosphamide, a known leukemogenic drug,[7,12] although there were no patients with secondary leukemia in this cohort of 35 patients, of whom five survived at least 1 year.

Eligibility criteria included: histologically confirmed NSCLC of stage IIIM$_1$, or stage IIIM$_0$ with either malignant pleural effusion or T$_3$ lesions with mediastinal involvement; no prior chemotherapy; creatinine clearance >70 mL/min; and a Zubrod performance status ≤3. All patients were advised of procedures and attendant risk, and gave informed consent, in accordance with federal and institutional guidelines. Patients were hospitalized for administration of cisplatin to ensure adequate hydration. Cisplatin was administered over six to eight hours with mannitol diuresis. Etoposide and vindesine were administered in the outpatient clinic, using a 30 to 60

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Table 1. Dose Schedules for VP, EP, VEP Regimens

Regimen	Drugs	Dose	Schedule and Route
VP	Vindesine	3 mg/m^2	Weekly IV for 12 weeks, then biweekly
	Cisplatin	120 mg/m^2	Every 3 weeks IV for 12 weeks, then every 6 weeks
EP	Etoposide	300 mg/m^2	Weekly IV for 12 weeks, then biweekly
	Cisplatin	120 mg/m^2	As per VP
VEP	Vindesine	3 mg/m^2	As per VP
	Etoposide	300 mg/m^2	As per EP
	Cisplatin	120 mg/m^2	As per VP and EP

Abbreviation: IV, intravenously.

minute infusion for etoposide and a rapid bolus for vindesine. Patients received at least two cycles (6 weeks) of therapy before re-evaluation. The therapy was discontinued in the event of progressive disease, unacceptable toxicity, or patient request. Palliative radiotherapy was allowed at any time.

Twenty-four patients survived for more than 1 year after initiation of therapy (either chemotherapy or radiotherapy), and these patients were analyzed further for risk factors for ANLL. Patients who survived <1 year were not considered to be at risk of developing ANLL because the reported latent period for this complication is rarely this short.[7,9,12,20] (In fact, none of these 95 patients were noted to develop ANLL.) The observation period was defined as time from start of therapy to death, ANLL (or the preleukemic phase), or September 18, 1986. The risk period was defined as the observation period following the first year. Risk estimates were determined by dividing the number of failures by the total person-years of risk, and an asymptotic maximum likelihood estimation was used to determine 95% confidence limits.[21] The cumulative risk of ANLL was also estimated according to the method of Kaplan and Meier.[22] The Mann-Whitney test was used to detect differences between the leukemic and nonleukemic patients.

RESULTS

Of the 24 1-year survivors, 17 were men and seven were women. The median age was 56 years (range, 38 to 69 years). Eighteen patients had metastatic disease, and six were stage IIIM$_0$. Twelve patients had adenocarcinoma, eight patients had squamous cell carcinoma, and four patients had large cell undifferentiated carcinoma. Three patients received only vindesine and cisplatin, nine patients only etoposide and cisplatin, and 12 patients received all three drugs. Nineteen patients received radiotherapy to limited fields, usually in the thorax. Only two patients received pelvic radiation. The median dose in the 19 patients was 30 Gy (range, 12 to 60 Gy).

Nineteen patients died after a median survival of 15 months (range, 12 to 26 months) without evidence of ANLL, one patient is alive (for 32 months) without evidence of ANLL, and four patients developed ANLL. The clinical and pathologic features of the latter group are summarized in Table 2, and their individual case reports follow.

Patient No. 1

A 57-year-old woman presented in September 1981 with bilateral pulmonary nodules. Bronchoscopy was positive for adenocarcinoma. In November 1981, she began therapy with cisplatin and etoposide. A partial response was documented in December 1981. Cisplatin was discontinued in April 1983 due to mild renal insufficiency, but etoposide was continued until March 1984. Blood counts at that time were hematocrit 22%, WBC count 3,700/μL, and platelets 65,000/μL. In April 1984, bone marrow aspiration and biopsy demonstrated a normocellular marrow with myelodysplasia but <5% myeloblasts. She was treated with cis-retinoic acid for 3 months without improvement. A second bone marrow examination in November 1984 was hypercellular with trilineage dysplasia and 25% to 30% myeloblasts. Cytogenetic analysis demonstrated a mosaic female karyotype characterized by three related abnormal clones, each with abnormalities of chromosomes 5 and 7 (Table 2). No further therapy was administered, and the patient died in November 1984.

Patient No. 2

A 58-year-old woman presented in May 1982 with a right lung mass and pleural effusion. Sputum cytology and thoracentesis were positive for adenocarcinoma. A bone scan showed increased uptake in the thoracic spine and right femur consistent with metastases. In June 1982, she began receiving VEP therapy with stabilization of her disease. In December 1983, she presented with dementia and ataxia. Lumbar puncture revealed malignant cells consistent with adenocarcinoma in the spinal fluid. An Ommaya shunt was implanted, and intraventricular methotrexate therapy (with oral

Table 2. Summary of Clinical, Morphologic, and Cytogenetic Features of Leukemic Patients

			Cumulative Dose (mg/m^2)*					
Patient	Age/Sex	Radiotherapy	VDS	VP16	DDP	Time to ANLL (mo)	Morphologic Classification	Karyotype
1	57/F	No	0	7,350	603	28	t-ANLL	46,XX(22%)/45,XX, −5, −7, +der (5)t(5;7)(q12;p11) (56%)/45,XX, same, del(12)(p11p13) (11%)/ 45,XX, −5, −7, +der(5),t(17;?) (q25;?)(11%)
2	58/F	No	77	7,950	590	19	AMoL	46,XX(27%)/ 46,X,t(X;10)(p11;p11)(70%) SCA:46,X,t(X;10),t(17;?)(q2?2;?)(3%)
3	59/M	Yes	35	4,382	120	13	AMoL	46,XY(53%)/ 46,XY,t(9;11)(p22;q23)(47%)
4	70/F	No	0	6,240	120	35	AMMoL	46,XX(5%)/46,XX,t (9;11;18)(p22;q23;q12)(95%)

Abbreviations: VDS, vindesine; VP16, etoposide; DDP, cisplatin; t-ANLL, therapy-related acute nonlymphocytic leukemia; AMoL, acute monoblastic leukemia; AMMoL, acute myelomonocytic leukemia; SCA, single cell abnormality.

*First year only.

folinic acid) was administered weekly. In January 1984, her WBC count was noted to be 64,000/μL, mostly monoblasts. The bone marrow was hypercellular with ~80% monoblasts and promonocytes, consistent with AMoL. Cytochemical staining supported the monocytic origin of the leukemic cells, as the alpha naphthyl esterase reaction was strongly positive in 89% of the marrow blasts and was completely inhibited by sodium fluoride. Cytogenetic analysis demonstrated a reciprocal translocation of the short arms of chromosome 10 and the X chromosome (Table 2). Treatment was begun with low-dose subcutaneous cytosine arabinoside (10 mg/m^2 twice daily), but the patient died ten days later of acute renal failure.

Patient No. 3

A 59-year-old man presented in February 1984 with a left pleural effusion. Thoracentesis and pleural biopsy were both positive for adenocarcinoma. Radiotherapy to the left hemithorax (30 Gy in 15 fractions) was administered in March 1984. He began receiving VEP therapy in April 1984, which was complicated by acute renal failure. In May 1984, therapy was restarted using only etoposide and vindesine. He continued without evidence of disease progression until January 1985 when a complete spinal cord compression (at T-8) was diagnosed. A laminectectomy was performed, followed by an additional 20 Gy (10 fractions) to the thoracic spine. In April 1985, the patient complained of severe fatigue, and a complete blood count revealed a hematocrit of 21%, WBC count of 1,100/μL, and a platelet count of 218,000/μL. A bone marrow biopsy demonstrated total replacement of the marrow by malignant cells, initially felt to be metastatic adenocarcinoma, but later confirmed as poorly-differentiated AMoL. Cytogenetic analysis demonstrated a reciprocal translocation involving the short arm of chromosome 9 and the long arm of chromosome 11 (Table 2). The patient was treated with cyclophosphamide 1,000 mg/m^2 and doxorubicin 40 mg/m^2 monthly for four cycles, and had no residual leukemia in September 1985. A follow-up bone marrow exam 2 months later also showed no evidence of leukemia, but the patient died of progressive carcinoma in January 1986.

Patient No. 4

A 70-year-old woman presented in August 1983 with a right lung mass. Needle aspiration of the mass was positive for adenocarcinoma. Chest computed tomography (CT) scan demonstrated bilateral hilar and mediastinal adenopathy, a right middle lobe mass, and an enlarged left adrenal gland. In October 1983 she was begun on etoposide and cisplatin but received only one dose of cisplatin secondary to deteriorating renal function. She continued to receive etoposide until August 1985, at which time disease progression was noted. In September 1986, her blood counts were hemoglobin 7.5 gm/dL, WBC count 1,900/μL and platelets 58,000/μL. The bone marrow was hypercellular with 50% blasts. Cytochemical staining of the marrow showed that 41% of the blasts were positive for alpha naphthyl esterase and 27% were positive for alpha naphthyl butyrate esterase, consistent with acute myelomonocytic leukemia. Cytogenetic analysis demonstrated a complex reciprocal translocation involving the short arm of chromosome 9, the long arm of chromosome 11, and the long arm of chromosome 18 (Table 2). The patient refused further therapy and died 1 month later.

Risk of ANLL

The 24 patients in this study population were at risk for a total of 13.4 person-years (after the first year) before death, ANLL, or this report. This yields a rate of ANLL of 0.30 per person-year, with 95% confidence limits of 0.11 to 0.80. In comparison, the rate of death from bronchogenic carcinoma during the same period was 1.42 per person-year (95% confidence limits are 0.91 to 2.23). The cumulative risk of ANLL by Kaplan-Meier analysis was 15% ± 11% at 2 years and 44% ± 24% at 2.5 years (Fig 1).

Comparison of Leukemic and Nonleukemic Patients

Nonparametric testing (Mann-Whitney) was used to compare the leukemic (n = 4) and nonleukemic patients (n = 20). There were no significant differences in the cumulative vindesine or cisplatin doses during the first year of observation (Table 3). However, the patients who eventually developed ANLL had received significantly more etoposide than the nonleukemic patients (Table 3, Fig 2).

DISCUSSION

This is the first report of ANLL following combination chemotherapy with cisplatin and etoposide. None of the leukemic patients ever received classical alkylating agents that have previously been associated with the development of t-ANLL.[3,4,7,12] Also, radiotherapy does not appear to be causative in these cases, since only patient no. 3 received radiotherapy and only to a limited thoracic port, not to the pelvis as has previously been associated with t-ANLL.[9]

Our circumstantial data suggest that high cumulative doses of etoposide may be causative in the development of ANLL. All four leukemic patients received both cisplatin and etoposide, but patients no. 3 and 4 received only a single dose of cisplatin because of nephrotoxicity. In fact, this allowed these patients to receive higher doses of etoposide. As shown in Fig 2, there appears to be a relationship between the total dose of etoposide during the first year of therapy and the development of ANLL. Other reports have suggested a similar dose effect for the alkylating agents.[3,12,23] However, we cannot exclude the possibility that the other known leukemogenic factors present in these patients (ie, smoking, radiation therapy, cisplatin), or even unknown factors such as the Tween 80 required for formulation of the etoposide, were contributory to the development of ANLL, especially in patients no. 2, 3, and 4.

The risk of ANLL in our patient population probably exceeds 11% per person per year with a cumulative risk of 44% ± 24% at 2.5 years, which is much higher than that

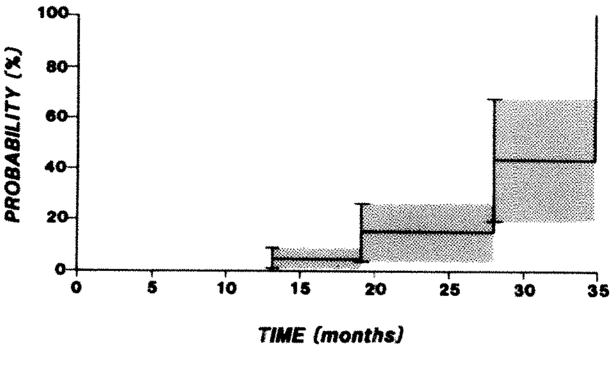

Fig 1. Cumulative risk of secondary ANLL in 119 patients (24 1-year survivors) treated for NSCLC with cisplatin-containing regimens (Kaplan-Meier estimate). Vertical markings represent ± SE.

Table 3. Comparison of Leukemic and Nonleukemic Patients

		Median Cumulative Dose (mg/m²)*		
Group	n	VDS	VP16	DDP
Leukemic	4	21 (0-77)†	6,795 (4,382-7,950)‡	355 (120-603)
Nonleukemic	20	17 (0-77)	3,025 (0-8,155)‡	485 (120-727)

Abbreviations: VDS, vindesine; VP16, etoposide; DDP, cisplatin.

*First year only.

†Range.

‡$P < .01$ (Mann-Whitney).

calculated in previous reports of t-ANLL. For example, Pederson-Bjergaard et al[7] estimated a risk of 1% to 1.5% per year after reviewing ~2,000 patients treated with alkylating agents for a variety of tumors. Greene et al recently assessed leukemogenic risk factors in women treated for ovarian carcinoma.[12] The overall 10-year risk was 8.5% ± 1.6%, with a cumulative risk of 19.5% ± 4.9% at 10 years in women who received >27,000 mg of melphalan, the subset at highest risk.

Although cisplatin and etoposide are often used in the management of germ cell tumors and small cell carcinoma of the lung,[24,25] there have been no reports of this combination being leukemogenic. This may be due either to differences in scheduling, or to the relatively low cumulative doses of etoposide received by these patients. In four 3-week cycles of cisplatin/etoposide as usually administered to patients with germ cell tumors, only 2,000 mg/m² etoposide is administered, using 100 mg/m² daily doses. Similar schedules have been used in small cell carcinoma,[25] with etoposide doses of 300 to 500 mg/m² per 3- to 4-week cycle. Thus, it is quite possible that the high incidence of ANLL in our patients is due to the unusual schedule and dose intensity of etoposide used in this study (Table 1). Our four leukemic patients received ~4,000 to 8,000 mg/m² of etoposide in 1 year, and the nonleukemic patients received a median of ~3,000 mg/m² for their NSCLC, usually 300 mg/m² per week.

Etoposide-induced ANLL may represent a new syndrome that differs from classical t-ANLL.[9,13] Although the clinical features of three of these patients raise the question whether they actually represent ANLL de novo, this is statistically unlikely. Patient no. 1 had trilineage dysplasia and the cytogenetic abnormalities of chromosomes 5 and 7 frequently observed in t-ANLL, but the other three patients had the morphologic and/or cytogenetic characteristics usually seen with AMoL. Moreover, patients no. 2, 3, and 4 lacked a preleukemic phase, which was noted in 76% of the 63 patients with t-ANLL reported by Le Beau et al.[9] Finally, the latency period from the start of therapy to overt leukemia in patients no. 2 and 3 was short, ie, 13 and 19 months. This compares with a median of 58 months in the study of Michels et al[8] and to a median of 56 months in the 63 patients with t-ANLL in our previous series.[9] However, Pedersen-Bjergaard et al recently reported four patients (of six) with small cell carcinoma of the lung who developed ANLL after a latency period of <2 years.[6]

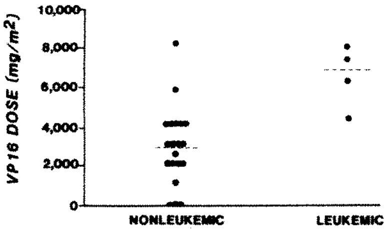

Fig 2. **Comparison of cumulative etoposide doses received by the leukemic and nonleukemic patients. The median cumulative etoposide dose (indicated by the bar) was significantly higher in the leukemic patients ($P < .01$, Mann-Whitney). The sole nonleukemic patient alive without ANLL received 2,005 mg/m² of etoposide. (VP-16, etoposide).**

As noted earlier, most patients with t-ANLL secondary to alkylating agents have losses of all or part of the long arm of chromosomes 5 and 7, and these abnormalities were present in patient no. 1. Two of our patients, however, had chromosome abnormalities characteristic of AMoL, namely translocations involving chromosomes 9 and 11, with breakpoints at 9p22 and 11q23.[26] AMoL has rarely been observed as a therapy-related leukemia; it did not occur in any of the 26 cases reported on by Rowley et al.[11] It is of interest that we have also studied one other patient who developed secondary AMoL, but she had been treated for ovarian carcinoma with alkylating agents. Michels et al noted two cases of AMoL in their series of 65 patients with secondary leukemia.[8] Two other patients with a secondary AMoL and a t(9;11) have been reported on separately by Weh et al[27] and Dewald et al.[28] The child described in the former report received combination chemotherapy including teniposide, another epipodophyllotoxin and a topoisomerase II inhibitor,[29] and cisplatin, and AMoL was diagnosed 38 months after beginning therapy; there was no preleukemic phase. The patient reported on by Dewald et al[28] also did not have a preleukemic phase before presentation with AMoL 7 years after therapy.

Weh et al proposed that acute leukemias with a specific translocation be considered primary, not secondary, leukemias.[27] This recommendation is based on their concern that such patients might otherwise not be treated in view of the generally poor response of patients with t-ANLL to therapy. Three of our own patients plus the other five cases noted had features usually associated with AMoL de novo, and these leukemias may have developed independent of the prior cytotoxic therapy received. A similar argument may be made for three other patients whom we have studied, each of whom developed acute promyelocytic leukemia with a t(15;17); two had received radiotherapy only, neither had a preleukemic phase, and the latent period was 23 and 56 months.[9] However, it is equally plausible that these specific chromosomal rearrangements were induced by mutagenic treatment, and that once these genetic rearrangements had occurred, the subsequent leukemia manifest the same clinical features typically seen when that particular chromosomal abnormality occurs spontaneously. For reasons not yet clear, it seems likely that high doses of etoposide, and possibly teniposide,[27] may induce genetic mutations that result in acute leukemia with a monoblastic phenotype.

The mechanism for malignant transformation by etoposide may differ from that of alkylating agents due to the different molecular pharmacology of these drugs. Alkylating agents are cytotoxic because of their ability to form cross-links with DNA.[30] Etoposide also interacts with DNA, by forming a ternary complex with topoisomerase II, which blocks the catenation activity of the enzyme leading to DNA strand breakage.[29,31] It has also been suggested that etoposide may cause DNA damage via a dehydrogenase-induced free radical intermediate.[32] In addition, it is possible that cisplatin may have contributed to the leukemogenesis in these patients, since it also acts to cross-link DNA.[33]

Although etoposide has not previously been demonstrated to be leukemogenic in humans, the topoisomerase II inhibitors are extremely potent clastogens in vitro, producing a high frequency of sister chromatid exchange and other chromosomal aberrations.[34-37] The mutagenicity of the closely related drug teniposide appears to be highly site-specific,[37,38] and thus teniposide-induced (or etoposide-induced) neoplasms might produce a unique clinical syndrome. Oncologists must be alert to the fact that increasing numbers of patients are being exposed to very potent mutagenic chemicals in an effort to treat malignant diseases effectively. The careful description of untoward consequences of some of these drugs, especially when used at high doses, will help to minimize these consequences. Thorough investigation of the few patients who unfortunately develop leukemia will provide critical insights into the mechanisms associated with leukemogenesis.

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NOTE ADDED IN PROOF

Fenaux et al[39] recently reported a case of AMoL following combination chemotherapy for osteosarcoma, associated with t(9;11)(p21;q23). This case also had a short latency period of 11 months, but the patient did not receive either etoposide or cisplatin.

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B Lymphocyte Precursors and Myeloid Progenitors Survive in Diffusion Chamber Cultures but B Cell Differentiation Requires Close Association With Stromal Cells

By Philip C. Kierney and Kenneth Dorshkind

The aim of this study was to investigate the relative contribution of direct contact with stromal cells *v* stromal cell-derived soluble mediators to the differentiation of B lymphocytes and cells from other hematopoietic lineages. This was investigated by making a comparison between hemopoietic cells grown in direct contact with stroma to those in diffusion chambers (DCs) placed over purified populations of stroma. The source of stromal cells was adherent layers from myeloid or lymphoid long-term bone marrow cultures that had been treated with mycophenolic acid, an antibiotic that depletes hemopoietic cells from the cultures but retains a functional stroma. The cells seeded into the chambers were fresh marrow cells that had been passed through two consecutive nylon wool columns to deplete cell populations capable of forming an adherent cell layer in vitro. DCs were placed in wells in which the adherent stroma, growing under myeloid or lymphoid conditions, was present. The results indicate that progenitors of granulocytes and macrophages survived and differentiated in DCs under myeloid culture conditions, as the number of cells and absolute number of CFU-GM increased over that present in the reseed population. These levels, however, were markedly less than in parallel cultures in which the cells were seeded directly onto stroma. Hematopoiesis in DCs placed over hemopoietically active stroma was not optimal, suggesting that factors were used by those hemopoietic cells closest to the stroma. A B lymphocyte precursor survived in DCs under myeloid but not lymphoid conditions, and its differentiation into B lymphocytes was dependent on close association with stromal cells; B lymphopoiesis initiated when cells from DCs grown under myeloid conditions were harvested from the chambers and seeded directly onto stroma initiated and maintained under lymphoid bone marrow culture conditions. B lymphopoiesis did not initiate if the DC from the myeloid conditions was left intact and placed directly over a lymphoid stromal cell layer in lymphoid conditions.

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THE PRIMARY production of B lymphocytes occurs in the bone marrow in postnatal life.[1-3] B lymphopoiesis, as well as the development of myeloid and erythroid cells, proceeds in the medullary cavity in association with a supporting population of stromal cells that creates a hematopoietic microenvironment that is apparently obligate for blood cell formation.[4-6] Although stromal cells are generally accepted to play a regulatory role in hematopoiesis, how they mediate their regulatory effects on developing B lineage cells remains incompletely understood.

Substantial evidence from both in vivo and in vitro morphological studies shows that direct stromal cell–hemopoietic cell contact is important, and associations between B cells and stromal cell processes have been observed using immunoelectronmicroscopic techniques.[7] Intracellular contacts resembling gap junctions have been suggested to be of possible importance in transmitting signals from the stroma to blood cells[8,9] although this has not been formally demonstrated in the development of B lymphocytes. Results from different long-term bone marrow culture systems, in which long-term hematopoiesis is dependent on the formation of a stromal cell adherent layer,[10] also suggest a role for direct contact in B lymphopoiesis. The myeloid bone marrow culture system (MBMC) of Dexter and colleagues is optimal for the growth of myeloid precursors and their differentiation into mature end cells.[11] B lymphocytes and their immediate precursors are not present in the cultures, but an early B cell precursor is present.[12,13] A higher proportion of these precursors,[12] as well as other progenitor populations,[14] is present in association with the adherent layer as compared with the nonadherent populations in the cultures.[12,14] Blood cells in the adherent layer of the MBMC are often covered by epithelial-like cells,[15] and such close interactions may also be observed in the lymphoid bone marrow culture system (LBMC) described by Whitlock and Witte.[16] B lymphopoiesis proceeds in those cultures but not myelopoiesis.[16] The lymphoid cells in the LBMC can often be observed to be enveloped by cytoplasmic infoldings of a single stromal cell.[17] Hemopoiesis is clearly also regulated by various hemopoietic growth factors,[18] some of which are produced by stromal cells.[19-22] Several laboratories have now isolated stromal cell lines that support proliferation and/or differentiation of B lineage cells. Some of these lines mediate their functions in part through secretion of soluble mediators[23-25].

Possibly only selected stages of B lymphopoiesis proceed under the influence of stromal cell-derived growth factors alone, and close stromal cell interactions may be required at certain points in that differentiative process. Distinguishing between these alternatives could be important in defining the signals that regulate B cell production from primitive hematopoietic precursors. This issue was investigated by comparing the growth of B lineage cells grown in direct contact with stromal cells to those seeded in diffusion chamber (DC) cultures placed over purified populations of stromal cells.

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The results indicate that B cell precursors, as well as myeloid progenitors and their differentiated progeny, can survive in DCs but that hematopoiesis is not as optimal as when direct stromal cell contact is permitted. The data further indicate that the differentiation of the MBMC B cell precursor into B lymphocytes only occurs when direct stromal cell contact is permitted.

MATERIALS AND METHODS

Animals. Six- to 8-week-old male or female BALB/cAn mice were produced in the vivarium of the Division of Biomedical Sciences, University of California, Riverside, from the breeding stock obtained from Life Sciences Laboratories, St. Petersburg, FL.

Preparation of cell suspensions. Mice were killed by cervical dislocation, and femurs were removed and placed in α-minimal essential medium (α-MEM; GIBCO, Grand Island, NY). Single cell suspensions of bone marrow were obtained by flushing the plug from bones with 3 mL medium. Cell viability was determined by eosin dye exclusion, and all cell counts were performed in a hemocytometer.

Generation of stromal cell cultures and stromal cell lines. Stromal cell cultures were generated by establishing long-term bone marrow cultures under myeloid or lymphoid conditions and subsequently treating these with the antibiotic mycophenolic acid, MPA, GIBCO). As previously described in detail, this procedure results in the depletion of hemopoietic cells while retaining a viable, functional adherent layer of stromal cells.[22] Purified stromal cells initiated under myeloid conditions were established by plating 4 mL of a bone marrow cell suspension at 10^6 cells/mL in α-MEM supplemented with 20% horse serum (GIBCO) and 10^{-6} mol/L hydrocortisone succinate (Upjohn, Kalamazoo, MI) in each 9.5-cm^2 well of a six-well plate (Costar, Cambridge, MA). Cultures were placed in a 33°C incubator and wrapped in Saran Wrap to prevent drying of cultures. Cultures were fed after 1 week, and by 2 weeks a confluent, adherent layer of cells had been established. At that time, all culture medium was removed from the wells and replaced with the above medium additionally supplemented with 5 μg/mL MPA. Three days later, all the MPA-containing medium was replaced by fresh medium and, following a 3-day incubation, the cycle was repeated. Purified stromal cells initiated under lymphoid conditions were similarly established as above except that the medium consisted of RPMI 1640 supplemented with 5% fetal calf serum (FCS) (GIBCO), and 5×10^{-5} mol/L 2-mercaptoethanol (2-ME), and all incubations were carried out in a 5% CO_2 and air incubator at 37°C. Cultures were fed once a week in their respective medium.

The preparation of stromal cell lines and their characteristics has been described in detail.[23] One of these, S17, was isolated from a myeloid bone marrow culture adherent layer and has the capacity to support B cell precursors and their differentiation into surface IgM expressing B lymphocytes. This line was grown to confluency in 10-cm^2 dishes and α-MEM plus 15% FCS. Cells from these dishes were harvested by trypsinization and subcultured in each 9.5-cm^2 well of a six-well plate (Costar).

Separation of cells on nylon wool columns. Cells from fresh bone marrow were passed through nylon wool columns prepared according to the method of Julius et al.[26] As previously demonstrated, this procedure depletes stromal cells capable of forming an adherent layer in vitro but retains hemopoietic precursors.[22] A maximum of 5×10^7 fresh bone marrow cells in a 2-mL vol was loaded onto a column packed in a 12-cc syringe and washed into the nylon wool with 1 mL 37°C medium. The nonadherent cells were eluted from the column by washing with 20 mL of the above medium. The eluted cells were then passed through a second column as described above. Cells were incubated 45 minutes on each column. Approximately 7% of the initial cell input remained following passage through the second nylon wool column. To confirm the efficacy of the separation procedure, aliquots of 5×10^5 of cells passed twice through nylon wool were cultured alone in tissue culture wells and observed throughout the course of the experiment for growth of stromal or hematopoietic populations.

DC cultures. DCs were seeded with bone marrow cells that had been passed through nylon wool and placed in the wells of the cluster dishes. The chambers, suspended in each well, consisted of a low protein binding, non-tissue culture-treated nucleopore membrane with 0.4 μ pore size (Costar). The distance between the bottom of the DC membrane and the bottom of the tissue culture well is 1 mm, thus minimizing the chance that stromal cell processes could have made contact with cells in the DC through pores in the DC membrane. Cells (10^5 and passed twice through nylon wool) were seeded into the chambers, which were then placed in the tissue culture wells with preestablished stromal layers. The cultures contained a total volume of 4.1 mL, with 1.5 mL contained within the DC and an additional 2.6 mL in the well below. Cultures were fed by first removing 0.75 mL from the chamber. This chamber was then removed from the well with sterile forceps, and one-half the volume was removed from the underlying well and replaced with fresh medium. The DC was then placed back in the well, and 0.75 mL of additional fresh medium was added to it.

Cultures under MBMC conditions were maintained at 33°C in α-MEM supplemented with 20% horse serum (GIBCO) and 10^{-7} mol/L hydrocortisone succinate (Upjohn). The cluster dishes were wrapped in Saran Wrap to prevent dessication because the 33°C 5% CO_2 and air incubator was not humidified. Cultures under lymphoid conditions were maintained at 37°C in a humidified 5% CO_2 and air incubator in RPMI 1640 supplemented with 5% FCS (GIBCO) and 5×10^{-5} mol/L 2-ME.

Transfer of cultures from myeloid to lymphoid conditions. In some experiments, cultures were transferred from myeloid to lymphoid growth conditions. For those cells grown in DCs under myeloid conditions, cells were harvested, washed once, and placed in a new diffusion chamber in 1.5 mL RPMI 1640, 5% FCS, and 5×10^{-5} mol/L 2-ME. This was then transferred to the 9.5-cm^2 well in which stroma grown under lymphoid conditions was present, containing 2.6 mL of the above medium at 37°C. Alternatively, aliquots of cells from the DCs were seeded directly onto stroma in LBMC conditions. Those myeloid cultures in which nylon-wool-passed cells had been seeded directly onto stroma were transferred by pooling the nonadherent cells with the loosely adherent ones obtained by vigorously washing the wells by repeated pipetting. After one wash, these cells were then reseeded onto adherent stromal cell layers established under lymphoid conditions.

Antibody labeling of cells and fluorescence microscopy. Surface IgM-bearing cells were identified with an affinity-purified, fluorescein-conjugated, goat anti-mouse IgM antibody (Southern Biotechnology, Birmingham, AL). This reagent was diluted 1:5 in α-MEM supplemented with 5% FCS and 1 mg/mL sodium azide. Aliquots of 1 to 2×10^6 cells harvested from cultures or fresh bone marrow were suspended in 100 μL diluted antiserum and incubated for 45 minutes on ice. Cells were then washed twice at 4°C in α-MEM without serum and resuspended in 0.2 mL α-MEM. To this was added an equivalent volume of 2% paraformaldehyde in phosphate-buffered saline (PBS). Cells were stored at 4°C until cell counts were performed on wet-mount preparations using a Leitz Laborlux microscope equipped for epifluorescence. At least 500 cells were counted per sample. Those cells that expressed the 14.8 surface antigen were identified by incubating 1 to 2×10^6 cells with 100 μL rat anti-mouse hybridoma supernatant (American-type culture collection, Rockville, MD) for 30 minutes at 4°C. Following two washes, cells were resuspended in 50 μL 1:5 dilution of an affinity-purified goat anti-rat Ig (Southern Biotechnology). No cells in fresh marrow or cultures stained with this reagent alone. After a 30-minute incubation at 4°C, cells were washed twice as above and resuspended in paraformaldehyde. Fresh bone marrow controls were included in all experiments and, on the average, 9% of cells in fresh marrow were surface IgM positive and 20% expressed the 14.8 antigen.

Myeloid colony assay. Myeloid progenitor cells capable of forming colonies in semisolid medium were assayed by culturing 5×10^4 cells in 35-mm plastic Petri dishes containing 1 mL methylcellulose medium.[27] The latter contained 0.8% methylcellulose, 30% FCS, 5×10^{-5} M 2-ME (Sigma, St Louis), and 10% medium conditioned by WEHI-3B (D^-) cells. This was prepared by culturing WEHI cells at 10^6 cells/mL in 40 mL α-MEM supplemented with 1% FCS. Following a 24-hour incubation, supernatants were collected, centrifuged, and stored at 4°C until used. Colonies were enumerated on day 8.

RESULTS

Hemopoietic cell survival in diffusion chamber under MBMC conditions. To determine how the growth of cells in diffusion chambers under MBMC conditions compared with that usually observed in primary MBMC, 10^5 bone marrow cells passed twice through nylon wool were seeded in DCs placed over purified stromal cell layers. The nylon wool passage depleted stromal cells that could potentially form a microenvironment within the DC, thus ensuring that survival of hemopoietic populations would be dependent on stromal cells in the underlying well. The source of this stroma was a primary MBMC that has been treated with MPA to deplete hematopoietic cells. This growth was compared with that in DCs placed over hemopoietically active stroma or following direct seeding of the nylon-wool-passed cells on purified stromal cell layers. The data in Table 1 show the results of three such experiments in which triplicate wells were evaluated for each condition.

Hematopoietic cells were consistently recovered following seeding in DCs placed over stromal cell layers obtained by MPA treatment. Three weeks following initiation of the cultures, the number of hematopoietic cells recovered from the chambers had increased three- to sevenfold over the number initially seeded. Morphological examination of these populations showed them to be primarily neutrophilic granulocytes and macrophages, a finding consistent with the fact that 70% to 90% of the cells expressed the MAC-1 surface antigen (data not shown). The data also indicate that progenitors of granulocytes and macrophages survived under DC conditions for the 3-week observation period as the absolute number of CFU-GM had increased up to fivefold over the number in the initial seed population. In no case was the absolute number of CFU-GM present 3 weeks postinitiation less than that present in the reseed population.

A second aliquot of the same nylon-wool-passed bone marrow cells was seeded directly onto MPA-derived stromal cell layers. As shown in the middle column of Table 1, a higher level of hematopoiesis was observed in these cultures, as the numbers of cells recovered had increased up to 33 times the initial number seeded. This was generally reflected in the number of CFU-GM present; in experiment 2, the numbers of these progenitors had increased to 91 times the numbers in the reseed population.

A third aliquot of the above cells was seeded into DCs placed over hematopoietically active stroma. These cultures were established by simultaneously seeding 10^5 of the nylon-wool-passed bone marrow cells into DCs and directly onto the MPA stromal cell layers over which the chambers were placed. The use of reseeded, MPA-treated stroma in this third condition ensured that the stromal cells in all experiments were manipulated in a comparable manner. The number of hematopoietic cells recovered from these DCs 3 weeks postseeding had decreased twofold in one experiment and had doubled in the other two. These conditions were not optimal, however, since the numbers of CFU-GM present were always less than in the initial nylon-wool-passed reseed population.

In all experiments, aliquots of nylon-wool-passed cells were seeded directly into empty wells. No adherent layer formed under these conditions, and hematopoietic cells did not survive past 1 week. Neither did hematopoiesis initiate in DCs placed into wells in which no adherent stromal layer was present. Before they were used, MPA-treated stromal cell cultures were observed for 2 weeks following the last drug treatment to ensure that hematopoiesis did not initiate from drug-resistant precursors.

Survival of B cell precursors in DCs. An early B cell

Table 1. Survival of Hemopoietic Cells in DCs

Parameter 3 weeks Postinitiation	Experiment	Growth Conditions of Nylon-Wool-Passed Bone Marrow: DC Over MPA Stroma	Direct Seed on MPA Stroma	DC Over Hemopoietic Stroma
Cell No. (relative change over 10^5 seeded)	1	$0.3 \times 10^6 \pm 0.3$ (↑3)	$2.4 \times 10^6 \pm 0.8$ (↑24)	$0.04 \times 10^6 \pm 0.05$ (↓2)
	2	$0.5 \times 10^6 \pm 0.3$ (↑5)	$3.1 \times 10^6 \pm 1.7$ (↑31)	$0.18 \times 10^6 \pm 0.05$ (↑2)
	3	$0.7 \times 10^6 \pm 0.2$ (↑7)	$3.3 \times 10^6 \pm 2.6$ (↑33)	$0.20 \times 10^6 \pm 0.1$ (↑2)
CFU-GM/10^5	1	210 ± 165	148 ± 15	0
	2	32 ± 9	244 ± 59	18 ± 6
	3	39 ± 8	23 ± 7	12 ± 8
Absolute no. CFU-GM (relative change over number seeded)	1	630 (↑5)	3,552 (↑28)	0 (↓125)
	2	160 (↑2)	7,564 (↑91)	32 (↓2)
	3	273 (*θ*)	759 (↑3)	24 (↓12)

Nylon-wool-passed bone marrow (10^5) seeded into DCs placed over MPA-purified stroma, directly onto MPA stroma, or in DC over stroma on which 10^5 hematopoietic cells had been seeded in parallel. Number of CFU-GM/10^5 in reseed population in experiments 1, 2, and 3 were 125, 83 and 280, respectively. Each experiment represents triplicate cultures per condition. Numbers indicate mean ± SD.

precursor, but not pre-B or mature B cells, is present under MBMC conditions in which direct contact with the stroma is permitted,[12,13] and these cells can be induced to differentiate into mature B cells following transfer to LBMC conditions.[28,29] This is demonstrated by the data in Table 2 in which nylon-wool-passed cells were seeded onto stromal cell layers under MBMC conditions (condition 1, Table 2); 3 weeks later, the cells were harvested and replated on LBMC stroma under LBMC conditions. As shown in Table 2, cells that expressed surface IgM were induced by 4 weeks later.

To determine whether the MBMC B cell precursor also survived in DCs under MBMC conditions for 3 weeks, cells from cultures were either transferred to a new DC that was placed over a stromal cell adherent layer established by treating a primary LBMC with MPA (condition 2, Table 2) or harvested from the DCs in MBMC conditions and plated directly onto LBMC stroma (condition 3, Table 2). These cultures were then maintained at 37°C in LBMC medium. Less than 5×10^5 cells were recovered from DCs after 4 weeks in LBMC conditions, and no IgM-expressing cells were included among this population. This was consistent with morphological observations that demonstrated these cells to be granulocytes and macrophages. It is not clear why these myeloid cells survived under the LBMC conditions, but very low levels of colony-stimulating factors sufficient to allow this could have been secreted by the stromal cells under lymphoid conditions. A markedly different profile was observed in cultures in which cells had been seeded directly on stroma (condition 3). Up to 2×10^6 cells were recovered from these cultures, and up to 10% expressed surface IgM. Morphological examination demonstrated virtually all these cells to be lymphoid. These data indicate that a B cell precursor can survive in DCs under MBMC conditions but requires a close association with stromal cells to differentiate into surface IgM-positive B cells.

The adherent layers in LBMC conditions onto which DC cells were seeded were MPA-treated LBMC stroma. This left open the possibility that the sIgM-positive B cells detected could have arisen from MPA-resistant precursors present in the adherent layer. To ensure that the B cells were indeed derived from the DC cells, replicate experiments were performed in which 10^5 nylon-wool-passed cells were seeded into DCs over MPA-treated stroma under MBMC conditions. Following a 3-week incubation period, cells were harvested from the chambers and plated onto a confluent layer of S17, a stromal cell line shown to support B cell differentiation,[23] under LBMC conditions. Three separate experiments were performed, and the mean number of cells (± SD) recovered following 4 weeks in LBMC conditions was $2.1 \pm 0.6 \times 10^6$. Phenotypic analysis demonstrated that 48% ± 3.6% of these expressed the 14.8 antigen and 12.4 ± 7.3% were surface IgM positive. These data confirm that a B cell precursor is present in DCs and can differentiate into B cells when provided with the proper conditions.

Adherent populations of functional stroma do not establish in DCs. The conclusion that a B cell precursor survives under DC conditions is dependent on confirming that the nylon-wool-passed cells are not in contact with an adherent layer of functional stromal cells. That the nylon-wool-passed cells cultured alone do not form adherent layers and that the DC membranes are designed to prevent cell adherence suggest that this is the case. Nevertheless we considered it critical to examine the DC membrane to exclude the possibility that a functional adherent layer had established under the influence of soluble mediators produced by the stromal cells in the lower chamber.

Following 3 weeks of growth under MBMC conditions, nonadherent cells were removed from the DC growing over MPA-treated stroma in MBMC conditions. The membranes were gently washed and stained with Wright's stain. Examination of the membranes at 100x under a dissecting microscope indicated that a few foci of cells adherent to the membrane were observed in one-half of the experiments. These appeared to be macrophages, as they presented with a central nucleus and abundant cytoplasm. In view of this result, however, experiments were designed to establish whether populations of cells that could support hematopoiesis did indeed establish on these DC membranes. This was tested by placing the DC, seeded with 10^5 nylon-wool-passed cells, over a well in which MPA-derived stroma was growing under MBMC conditions. After 3 weeks, the nonadherent cells were removed from the chambers and the membranes were gently washed. The DCs were then placed over a well containing medium only in MBMC conditions and reseeded with 10^5 nylon-wool-passed cells. If a functional stromal layer had established in the DC, hematopoiesis would have been expected to initiate. Cultures were fed weekly and then examined for cell growth at 3 weeks postseeding, but no cells were recovered at that time. This experiment indicates that functional adherent layers do not establish in DCs under the experimental conditions used and suggests that the B cell precursor and CFU-GM that survive in DCs do so under the influence of soluble mediators secreted by stromal cells in the underlying well.

Table 2. Survival of B Cell Precursors in DC

Condition	Growth Under MBMC Conditions (3 weeks)	Following → Switch to	LBMC Conditions (4 weeks)	Cell No. After 4 Weeks in LBMC Conditions	IgM Positive (%)
1	Stroma	→	Stroma	$1 \times 10^6 \pm 0.1$	6.4 ± 5
2	DC	→	DC	$0.3 \times 10^6 \pm 0.15$	0
3	DC	→	Stroma	$1.4 \times 10^6 \pm 0.7$	11 ± 0.7

After 3 weeks under MBMC conditions (α-MEM, 20% horse serum, 10^{-6} mol/L steroids, 33°C), either on stroma directly or in DCs over MPA stroma layers, cultures switch to LBMC conditions (RPMI 1640, 5% FCS, 5×10^{-5} mol/L 2-ME, 37°C). Cells were left in contact with stroma, kept in DC, or removed from DC and placed directly on LBMC stroma. All data are based on two separate experiments in which three wells per condition were evaluated.

LBMC stromal cells do not support B cell precursors under DC conditions. The above experiments suggest that MBMC stromal cells produce soluble mediators that allow survival of B cell precursors in DC. Whether LBMC stromal cells can also support this precursor was tested by seeding 10^5 marrow cells passed twice through nylon wool into DCs placed over LBMC stromal cells in lymphoid conditions or directly onto the stroma. The data in Table 3 indicate that a close association with stromal cells is required for cell survival under LBMC conditions. Following 4 weeks of culture, B lymphopoiesis had not initiated and no hematopoietic cells were recovered from the DCs. Cells that expressed the 14.8 antigen and surface IgM were consistently recovered from parallel cultures in which the nylon-wool-passed cells had been directly seeded onto the stromal cell layer. These data establish that the initiation of B lymphopoiesis in LBMC requires a close association with the stroma and suggest that under LBMC conditions LBMC stromal cells do not secrete a soluble mediator that can support the B cell precursor under DC conditions.

DISCUSSION

The experiments reported herein used an in vitro DC culture system to evaluate the relative contribution of direct contact with stromal cells *v* the soluble mediators produced by them in the differentiation of B lymphocytes and other myeloid populations. The findings clearly indicate that, at least for the 3-week period of observation, hematopoiesis can proceed in DCs under MBMC conditions. Progenitors of granulocytes and macrophages were detected, and their numbers had increased over those present in the reseed population. This could indicate that self-renewal of these progenitors had occurred or that they were derived from an earlier cell through differentiation and amplification. The presence of morphologically identifiable granulocytes and macrophages in the chambers also suggests that differentiation of CFU-GM had occurred as well. A B lymphocyte precursor was detected in the DCs, but no conclusion can be drawn as to whether it is part of a stem cell pool or a restricted progenitor cell population. We must assume that the survival of these cell types was dependent on the production of soluble mediators from the underlying stroma; no cells survived in DCs placed in wells in which no stroma was present, and analysis of the DC membrane indicated that functional stroma had not established. A few adherent foci were observed, but these cells did not support hematopoiesis. Their identity has not been established, but they resemble macrophages morphologically.

Myeloid progenitors also survive upon implantation of DC in vivo into the peritoneal cavity of mice,[30,31] further substantiating the role of soluble mediators in the maintenance of selected hematopoietic populations. The factor-dependent survival of various long-term hematopoietic lines also substantiates this view. Greenberger and colleagues described a permanent progenitor line that was dependent on factor(s) present in WEHI-3–conditioned medium. This line was multipotent as it could generate neutrophils, basophils, mast cells, and erythroid cells.[32] Growth of factor-dependent cell lines was also reported by Dexter et al.[33] and Nagao et al.[34] Seemingly contradictory results from studies of long-term cultures from hamster marrow indicated that neither an adherent layer nor an exogenous factor addition was required for progenitor cell survival in vitro. As noted by those investigators, however, cell survival in that system may have been due to greater sensitivity to factors present in the serum used or the production of mediators by endogenous cell populations.[35]

Additional indirect evidence for the dependence of cells in DCs on soluble mediators was obtained in experiments in which hematopoietic cells were simultaneously seeded into DCs and onto the underlying stroma. Very few hemopoietic cells were recovered from DCs under those conditions, and progenitor cell numbers had declined markedly. That hematopoiesis had established in association with the adherent layer suggests that those hematopoietic cells closest to the stroma had used the factors produced by it, leaving only low amounts available to the cells in DCs. This may indicate that localized hematopoietic microenvironments exist in vivo and that mediators produced by the stromal cells were used by the hematopoietic cells closest to them.[36] Thus, although some mediators could potentially diffuse longer distances, they indeed may function as short-range molecules. This

Table 3. B Lymphopoiesis in Primary LBMC Requires Stromal Cell Contact

Parameter 4 Weeks Postinitiation	Growth Conditions of Nylon-Wool-Passed Bone Marrow			
	Experiment	DC Over MPA LBMC Stroma	Experiment	Direct Seed on MPA LBMC Stroma
Cell No. (relative change over 10^5 seeded)	1	0	1	$1.1 \times 10^6 \pm 0.5$ (↑11)
	2	0	2	$4 \times 10^5 \pm 0.2$ (↑4)
% 14.8 (relative change in absolute no.)	1	0	1	5 (↑5)
	2	0	2	36 (↑22)
% IgM (relative change in absolute no.)	1	0	1	1 (↑11)
	2	0	2	19.1 (↑76)

Nylon-wool-passed bone marrow (10^6) seeded into DCs placed over MPA-purified LBMC stroma or directly onto MPA-purified LBMC stroma under LBMC conditions. Assays were performed 4 weeks postseeding. Numbers indicate mean ± SD. Percentage of 14.8+ cells in experiments 1 and 2 in the NW reseed population were 10 and 6.5, respectively. Percentage of $sIgM^+$ cells in reseed population in experiments 1 and 2 in the NW reseed population were 1 and 0, respectively. Data expressed as mean ± SD. Each well (three per condition) counted individually, but cells were pooled for cell surface staining.

could further explain why it is difficult to detect factors in supernatant medium from ongoing long-term bone marrow cultures,[11] since all that is produced is bound by the hematopoietic populations in the cultures.

Although hematopoiesis did proceed in DCs under MBMC conditions, blood cell differentiation is not necessarily independent of close stromal cell associations. Indeed, the results we present argue that such associations are required, since the number of cells and progenitors was always greater in parallel cultures in which cells were seeded onto stroma. The inability of the DC B cell precursor to differentiate into B lymphocytes in DC under LBMC conditions further supports this position. These associations could be mediated by direct contact between hematopoietic and stromal cells. Alternatively, developing blood cells may require interactions with growth factors presented within the context of the hematopoietic microenvironment,[37] such as by their attachment to components of the extracellular matrix.[38] Thus, DCs may permit short-term, low-level hematopoiesis in the absence of optimal conditions. Nevertheless, the DC approach is useful for demonstrating the influence of factors on particular lineages and maturational states.

The demonstration of a B cell precursor in DCs makes this point particularly evident. A B cell precursor was present in DCs in MBMC medium 3 weeks postseeding. That mature B and pre-B cells are not present under these conditions[12] suggests that a factor that acts on B cell precursors exists. Whether this putative mediator promotes survival of these populations or their self-renewal cannot be determined from these experiments. How it might relate to the pre-B cell differentiation factor secreted by stromal cell line S17 is also unknown. The latter agent potentiates the expression of the 14.8 antigen and cytoplasmic μ heavy chains in bone marrow B cell progenitors in a 24-hour short-term assay.[39] Different mediators, whose actions affect distinct B lineage target cells, may exist. Ultimately, identification of the factor that maintains B cell precursors in DC will be dependent on replacing the underlying stroma with a conditioned medium that can be characterized.

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Clonogenic Hemopoietic Precursors in Bone Marrow Transplantation

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Multilineage and single-lineage hemopoietic precursors were studied in 102 bone marrow transplant recipients and their respective donors to determine their contribution to clinical outcome as measured by time to engraftment and survival. The patient population was heterogenous with respect to diagnosis and disease status. They included individuals with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), aplastic anemia, and a few other hematopoietic malignancies. The frequency of various clonogenic precursors in the normal donor population varied considerably. The data yielded a symmetrical distribution. In contrast, most bone marrow transplant recipients presented with significantly reduced numbers of clonogenic cells before transplantation, resulting in skewed distribution profiles. Serial studies of recipients demonstrated a significantly lower than normal level of clonogenic precursors even 3 and 4 years after transplantation. The median values and distribution profiles approximated those observed before transplantation but did not return to measurements obtained for normal donors. Patients with ALL deviated from this pattern. The median values and distribution profiles of clonogenic precursors before transplantation approximated the pattern of normal donors. The frequency of clonogenic progenitors after transplantation, however, remained significantly lower than that of their respective donor or pretransplant values. Cell cycle studies performed after normalization of peripheral blood hematopoietic parameters demonstrated for most recipients that a higher than normal proportion of multipotent cells was in S-phase ($P = .011$). By univariate and multivariate approaches, clonogenic precursors and clinical parameters were assessed for their contributions to clinical outcome as measured by time to engraftment and survival time. The number of nucleated cells in the transplant inoculum contributed to survival independent of other risk factors. Patients with a higher cell load had a higher probability of surviving than did patients with a lower cell concentration in the transplant inoculum ($P = .042$). The frequency of clonogenic precursors in the transplant inoculum altered neither survival nor time to engraftment. The time to engraftment was significantly influenced by the frequency of clonogenic megakaryocyte precursors (CFU-M) observed in recipients prior to transplantation ($P = .003$). Patients with high values engrafted faster than did patients with a low frequency of CFU-M. This was independent of both diagnosis and disease status of the patients at time of transplantation.

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CLINICAL BONE MARROW transplantation (BMT) has developed into a treatment modality with curative potential for patients with hematopoietic malignancies and other bone marrow deficiency disorders. Most long-term disease-free survivors are able to maintain production of peripheral blood cells in normal quantity. Despite this ability, the frequency of early hemopoietic precursors may not return to normal. This was suggested by studies reporting a low frequency of granulocyte-macrophage and erythroid progenitors in recipients after transplantation.[1,2]

The objective of this study was to monitor pluripotent precursors (CFU-GEMM) and progenitors committed to erythropoiesis (BFU-E), megakaryocytopoiesis (CFU-M), and granulocyte-macrophage production (CFU-C) in BMT recipients before and serially after transplantation as well as to assess their cell cycle status. In addition, the concentration of clonogenic cells was determined in each transplant inoculum. By univariate and multivariate approaches, the culture data were analyzed in conjunction with clinical parameters to determine their contribution to the outcome of the patients as measured by time to engraftment and survival time.

MATERIALS AND METHODS

Patients

One hundred two consecutive patients undergoing allogenic BMT at the Ontario Cancer Institute between March 1980 and January 1984 and their respective HLA-identical donors gave informed consent to be entered into the study (Table 1). The preparative regimens were described previously in detail.[3] In brief, patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) were prepared with a continuous infusion of cytosine arabinoside at a dose of 100 mg/m^2/day for 5 days, followed by cyclophosphamide at 60 mg/kg/day for 2 days and total body irradiation (TBI) at a mid-plane dose of 500 cGy delivered at 50 to 85 cGy/min by a single cobalt source.[4] Patients with acute lymphoblastic leukemia (ALL) received 2 mg Vincristine intravenously (IV), 200 mg of predisone/day for 5 days, 30,000 U L-Asparaginase per day for 5 days, cyclophosphamide 60 mg/kg/day for 2 days, and a single fraction of TBI at 500 cGy. Patients with aplastic anemia were treated with cyclophosphamide at 50 mg/kg/day for 4 days followed by a single dose of 300 cGy TBI. Attempts were made to transplant at least 3×10^8/kg nucleated cells of the recipient's body weight.

Two different consecutive regimens for graft-*v*-host disease (GvHD) prophylaxis were used. The first 34 patients were treated with the standard methotrexate regimen until day 100.[5] The following 68 patients received Methotrexate on days 1, 3, 6 and 11 and prednisone at a daily dose of 40 mg/m^2 starting on day 10. This dose was sustained until day 40 and subsequently tapered. Sixty-six patients transplanted from March 1980 to December 1982 who developed acute GvHD of grades II through IV were treated with

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Table 1. Patient Attributes

Diagnosis	Status	n	Median Age (yr)	Age Range (yr)	n Surviving	n Relapsing	Median* Follow-up (mos)
CML	CPI	14	29	17-37	7	0	36
	AP	17	34	19-45	5	1	45
	BC	5	32	19-40	0	2	22
	CP2	1	30	23-37	1	0	40
AML	1stR	19	31	17-42	11	2	28
	>1stR	8	22	16-40	1	4	23
ALL	1stR	9	23	17-37	6	2	43
	>1stR	10	21	18-33	4	3	47
AA	—	14	21	15-44	8	3*	45
Other†	—	5	30	27-39	2	2	27

Abbreviations: CPI, first chronic phase; AP, accelerated phase; BC, blast crisis; CP2, second chronic phase; 1stR, patients in first remission; >1stR, patients in relapse or subsequent remission.

*Graft rejection.

†Median follow-up of patients who are alive.

various regimens including high-dose predisone and/or antithymocyte globulin (ATG). Thirty-six patients transplanted since 1983 who developed acute GvHD received therapy with cyclosporine A (CyA). The initial dose of CyA was either 12.5 mg/kg/day orally or 5 mg/kg/day IV delivered in two divided doses at 12-hour intervals.[6] Subsequent doses were adjusted to maintain normal creatinine values and CyA levels between 50 and 300 μg/L. Patients with chronic GvHD were treated with azathioprine and prednisone. The median follow-up of the entire group was 38 months, with a minimal follow-up of 18 months at the date of the last follow-up.

Preparation of Cells

Bone marrow samples were obtained from transplant recipients prior to their ablative therapy and serially after transplantation on day 14, after 1, 2, 3, 6, and 12 months, and then annually. The samples were aspirated into heparinized syringes. The donor specimen consisted of 5 to 10 cc of bone marrow obtained as part of the transplant inoculum.

Cell Culture

Bone marrow samples were cultured as previously described[7,8] in Iscove's modified Dulbecco's medium (IDMEM, GIBCO, Grand Island, NY) supplemented with 0.9% methylcellulose (Dow Chemical, Midland, MI), 5×10^{-5} mol/L 2-mercaptoethanol (Sigma, St Louis), 5% medium conditioned by phytohemagglutinin-stimulated leukocytes (PHA-LCM, PHA, HA 15; Wellcome Diagnostics, Dartford, England), and 30% human plasma obtained from a patient with severe aplastic anemia. The latter contained an equivalent of 2 to 3 U/mL erythropoietin (EPO). The culture plates were scored after 14 days by inverted microscopy for the presence of multilineage colonies, erythroid bursts, and megakaryocyte and granulocyte-macrophage colonies.

Cell Cycle State Analysis

Bone marrow samples of 17 normal BMT donors and 29 BMT recipients at least 6 months post engraftment were examined to determine the proportion of clonogenic precursors in S-phase by preincubation with tritiated thymidine (^{3}HTdR) as described by Minden et al.[9]

Statistical Methods

Statistical analysis of counted data, such as colony numbers, was performed on the square-root transformation of the actual data value. This transformation resulted in a more symmetrical distribution than did the actual colony counts. It was also used when the prognostic value of colony counts was assessed in the Cox regression model described below. The Mann-Whitney test[10] was used for simple comparisons between groups. Pearson product-moment correlations[10] were computed on appropriately transformed data.

The prognostic value of cell culture and clinical parameters for time to engraftment and survival were evaluated by univariate and multivariate analysis. For the purpose of this study, survival time is defined as the time from BMT to death or to the date of last follow-up. Time to complete engraftment of all three hematopoietic lineages is defined as the time from BMT to the time when >50,000 platelets and reticulocytes and >1,500/μL neutrophilic granulocytes of peripheral blood were observed. Time to partial engraftment is defined as the time from BMT to the time when cells of at least two lineages were present in the above-stated frequency. Seventy-nine patients (77%) surpassed the stated lower limits for all three lineages, 95 (92%) surpassed the limits for at least two, and 100 (97%) surpassed the limits for at least one of the hemopoietic lineages. The analysis of time to complete or partial engraftment included only the respective subsets of patients who ultimately fulfilled the criteria of complete or partial engraftment as defined above. This approach was chosen to avoid violation of the statistical assumption that the time to engraftment was independent of the censoring time for engraftment (in this case, time to death).

The examined donor parameters included the number of nucleated cells and number of CFU-GEMM, BFU-E, CFU-M, and CFU-C in the transplant inoculum. In addition, these values were also expressed as cells transplanted per kilogram of the recipient's body weight. Each recipient was characterized by the frequency of these clonogenic precursors and the number of reticulocytes, platelets, and neutrophilic granulocytes prior to transplantation. In addition, clinical parameters of the recipient were taken into consideration. These included age, sex, weight, diagnosis, and risk as based on the disease status at the time of transplantation. The good-risk group included patients with AML and ALL in first remission, patients with CML in first chronic phase, and patients with aplastic anemia. The poor-risk group included patients with AML and ALL in relapse and subsequent remission, patients with CML in accelerated phase, blast crisis, or second chronic phase and the patients with multiple myeloma, lymphoma, and idiopathic thrombocythemia. The latter subgroup of various hemopoietic disorders was included since these patients presented with advanced disease. Each recipient was also assessed for the presence of GvHD.

The influence of each attribute was initially evaluated in a univariate analysis. Continuous variables were examined using the Cox regression method.[11] Categorical variables were assessed using log-rank (Savage) statistics.[12] Contributions to engraftment and survival by attributes that were significant were displayed by Kaplan-Meier plots.[13]

Multivariate analyses were performed to determine whether or not effects of cell culture parameters observed by univariate studies were sustained when tested in conjunction with clinical parameters. For this purpose, stratified and unstratified multipredictor Cox regression models[11] were constructed to control for the effects of prognostic factors.

RESULTS

Clinical Data

Details about age, diagnosis, and disease status of all patients at the time of transplantation are listed in Table 1. In addition, the number of surviving patients and the relapse rates are presented for each subgroup. The Kaplan-Meier plot of the overall survival of the whole group is shown in Fig

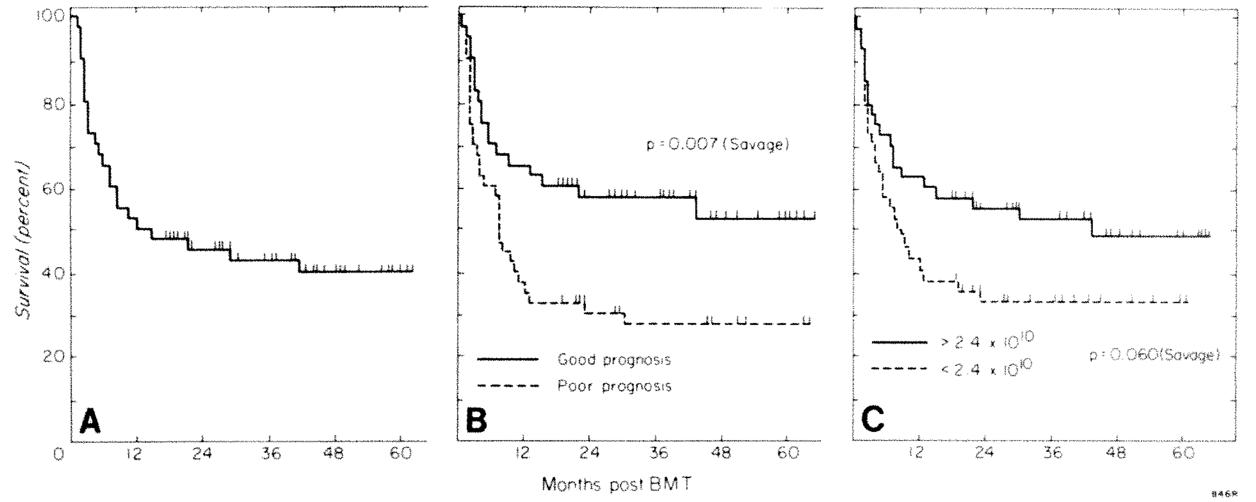

Fig 1. (A) Overall survival curve for all patients (Kaplan-Meier). (B) Survival curves for low-risk and high-risk disease as defined by disease status at the time of transplantation. (C) Survival curves of patients transplanted with less or more than 2.4 × 10^{10} mononuclear cells.

1A. The subgroup of 42 patients with early well-controlled leukemia included 19 with AML and 9 with ALL in first remission as well as 14 with CML in first chronic phase. Twenty-four (57%) are alive and free of relapse at the end of the study. Two patients with AML and two with ALL have relapsed. None of the surviving patients with CML have shown signs of disease recurrence.

The 41 patients with more advanced leukemia included 8 with AML and 10 with ALL in relapse or subsequent remission as well as 22 with CML who presented either in accelerated phase (17 patients), blast crisis (5 patients), or second chronic phase (1 patient). Nine patients are alive and have remained free of relapse (23%) (Fig 1B); ten (24%) have relapsed.

The small subgroup of 5 patients with other hematopoietic malignancies included 2 patients with lymphoma, 2 patients with multiple myeloma, and 1 patient with idiopathic thrombocythemia. One patient with lymphoma remains alive and disease-free; both patients with myeloma have relapsed; one is still alive on conservative therapy.

Eight of the 14 patients transplanted for severe aplastic anemia are alive; all have normal peripheral blood values. Forty-four of the entire group of 102 patients are alive (Fig 1A). The causes of death in the remaining 58 were related to recurrence of the original hematopoietic malignancy in 19%, to infections alone in 15%, and to GvHD alone in 12%. The majority (44%) succumbed to a combination of GvHD and infections. Other causes of death (8%) included poor engraftment or early rejection, severe hemorrhagic complications, and therapy-related central nervous system (CNS) toxicity. 19 patients had infections associated with development of a fatal interstitial pneumonitis. Three additional patients developed clinical and histologic features of bronchiolitis obliterans.

Bone Marrow Culture Studies

Clonogenic precursors in transplant inoculum. Each BMT inoculum was assessed for the number of mononuclear cells and the frequency of clonogenic hematopoietic precursors. The frequency of CFU-GEMM, BFU-E, CFU-M, and CFU-C per 2 × 10^5 mononuclear cells varied considerably from donor to donor. The distribution profiles for clonogenic cells of the donor population showed nearly symmetrical patterns with similar mean and median values. Median values are listed in Table 2. The culture data were used to calculate the number of clonogenic cells for each transplant inoculum and the number of clonogenic cells transplanted per kilogram of the recipient's body weight (Table 3). Again, a considerable patient to patient variation was observed.

Table 2. Clonogenic Hematopoietic Precursors of the Donor and Recipient Population Before and After BMT

Population	n	CFU-GEMM	BFU-E	CFU-M	CFU-C
Donors	100	12 ± 1*	159 ± 17	25 ± 6	127 ± 10
Recipients PRE-BMT					
All patients	80	2 ± 1	46 ± 22	3 ± 2	82 ± 12
CML	31	1 ± 1	44 ± 30	0 ± 1	128 ± 40
AML	18	4 ± 1	33 ± 21	8 ± 5	91 ± 19
ALL	16	8 ± 2	118 ± 28	23 ± 5	79 ± 12
AA	12	0 ± 0	0 ± 1	0 ± 0	0 ± 1
Other	5	6 ± 0	186 ± 0	33 ± 0	125 ± 0
Recipients Post-BMT					
1†	66	2 ± 1	18 ± 4	2 ± 2	37 ± 8
3	42	2 ± 1	38 ± 12	5 ± 1	43 ± 8
6	46	3 ± 1	43 ± 10	6 ± 1	44 ± 9
12	39	3 ± 1	69 ± 10	6 ± 1	55 ± 6
24	25	4 ± 1	54 ± 22	6 ± 4	49 ± 9
36	17	4 ± 1	35 ± 21	3 ± 3	53 ± 10
48	7	2 ± 7	70 ± 33	2 ± 3	54 ± 16
Recipients without continuing immunosuppression					
3	7	4 ± 1	51 ± 25	4 ± 2	63 ± 19
12	9	6 ± 2	53 ± 36	5 ± 2	62 ± 25
24	6	2 ± 2	19 ± 46	6 ± 18	47 ± 15
36	6	3 ± 3	42 ± 59	2 ± 13	65 ± 18

*Median SE of median for 2 × 10^5 mononuclear cells plated.

†Months.

Table 3. Frequency of Total Nucleated and Clonogenic Cells in the BMT Inoculum

Cell Populations	No. per Inoculum	No. per kg of Recipient's Body Weight
Nucleated	2.4×10^{10}*	3.7×10^{8}
	$(1.2\text{-}4.0 \times 10^{10})$†	$(1.8\text{-}5.9 \times 10^{8})$
CFU-GEMM	1.9×10^{6}	2.9×10^{4}
	$(0.3\text{-}8.5 \times 10^{6})$	$(0.4\text{-}14.8 \times 10^{4})$
BFU-E	24.4×10^{6}	35.2×10^{4}
	$(3.5\text{-}122.5 \times 10^{6})$	$(3.5\text{-}119.0 \times 10^{4})$
CFU-M	4.7×10^{6}	7.3×10^{4}
	$(0.7\text{-}24.5 \times 10^{6})$	$(1.2\text{-}42.0 \times 10^{4})$
CFU-C	16.5×10^{6}	25.9×10^{4}
	$(1.5\text{-}55.5 \times 10^{6})$	$(3.0\text{-}60.0 \times 10^{4})$

*Mean.

†Range.

Clonogenic cells in bone marrow samples of recipients before transplantation. Bone marrow samples of BMT recipients were obtained before administration of the preparative regimens and examined for their ability to form multi-lineage and single-lineage colonies. The distribution profiles for CFU-GEMM, BFU-E, CFU-M, and CFU-C were skewed toward lower colony frequencies. Their median values were significantly lower when compared with the respective values of the donor population (Table 2).

The data were also analyzed separately for each disease group. Patients with AML, CML, and aplastic anemia showed low median values for CFU-GEMM, BFU-E, and CFU-M (range of P values .00001 to .05, Mann-Whitney). The median CFU-C value was only significantly lower than controls for patients with aplastic anemia (P = .00001, Mann-Whitney). In contrast, median colony values for patients with ALL approximated counts of the normal donor population (range of P values .07 to .34, Mann-Whitney).

Clonogenic cells after transplantation. Only a few colonies were identified in bone marrow samples obtained 14 days after transplantation. Predominance of a specific colony type emerging early was not observed. In particular, the frequency of multilineage colonies was not increased in preference to single-lineage colonies. Sequential follow-up demonstrated an increase of all hemopoietic colony types. Normal colony numbers were only occasionally observed, however, even after follow-up periods of >3 years (Table 2). Colony formation in BMT samples obtained from most BMT recipients was reduced to 20% to 40% of values determined for the BMT donor population. The frequency distribution and median values of the entire group 1 year after transplantation approximated the median values of the recipients before transplantation (range of P values .11 to .84, Mann-Whitney). The values remained significantly lower when compared with the respective donor parameters (all P values = .00001, Mann-Whitney). The significant difference from donor values was confirmed when subgroups of patients with AML, CML, and ALL were analyzed individually (range of P values .0001 to .0052). The available sample size for patients with aplastic anemia was too small for an appropriate analysis. The values of clonogenic cells were reduced independent of continuing immunosuppressive therapy. This was demonstrated by examining the subgroup of ten patients that had not received any further immunosuppressive therapy after completion of their prophylactic GvHD regimens. The hematopoietic parameters of this subgroup and of their respective donors prior to transplantation were comparable to those of patients under further medication. The data are listed separately in Table 2 and do not show any difference when compared with those of the whole group.

Cell cycle analysis post-BMT. The proliferative status of precursor populations after transplantation was examined by assessing their cycle state using the ^{3}HTdR suicide technique (Fig 2) and compared with that of normal controls. In normal bone marrow specimens 65% to 95% of CFU-GEMM (median 80%) survived preincubation with ^{3}HTdR. In contrast, samples of BMT recipients tested after normalization of their peripheral counts yielded greater variability in the proportion of surviving CFU-GEMM. The values ranged from 8% to 100%, with a median of 60%. The observed distribution of values differed significantly from the normal control group (P = .011, Mann-Whitney). A similar observation was made for the committed progenitors BFU-E, CFU-M, and CFU-C. The proportion of pluripotent progenitors surviving a preincubation with ^{3}HTdR did not differ from the surviving fractions of committed precursors (range of P values .748 to .830).

The cellularity of the tested bone marrow samples as assessed by the number of mononuclear cells per milliliter of

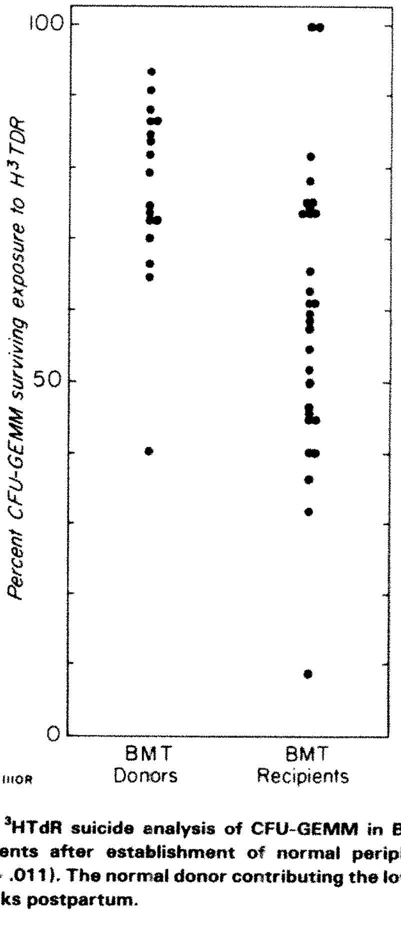

Fig 2. ^{3}HTdR suicide analysis of CFU-GEMM in BMT donors and recipients after establishment of normal peripheral blood values (P = .011). The normal donor contributing the lowest values was 4 weeks postpartum.

Table 4. Influence of Clonogenic Cells in BMT Inoculum and Bone Marrow Samples of the Recipients Before Transplantation

Attributes	Time to Engraftment	Survival
BMT Inoculum		
Nucleated cells	.654*	.042
CFU-GEMM	.801	.800
BFU-E	.590	.362
CFU-M	.390	.966
CFU-C	.564	.123
BMT Recipient		
CFU-GEMM	.225	.298
BFU-E	.269	.182
CFU-M	.003	.849
CFU-C	.809	.216
Reticulocytes	.200	.873
Neutrophils	.926	.694
Platelets	.842	.478
Age	.723	.081
Sex	.142	.223
Weight	.262	.533
Diagnosis	.254	.472
Disease status	.847	.006
GvHD	.009	.135

*P values of univariate analyses using a Cox regression analysis for continuous variables and Savage statistics for categorical values.

aspirate did not differ for the donor and recipient population ($P = .528$, Mann-Whitney).

These data confirm that nearly all available clonogenic cells are involved in the ongoing production of blood cells. Thus, bone marrow reestablished after BMT appears to function like a marrow under stress without displaying a reserve of resting precursors.

Analysis of Parameters Influencing Time to Engraftment

Time to engraftment. Culture parameters and clinical attributes were examined separately for their influence on time to engraftment. The respective P values are listed in Table 4. The presence of GVHD was the only clinical parameter significantly correlated with the time to engraftment. Patients without GvHD engrafted faster than did patients with GvHD ($P = .009$, Savage). The data are shown in Fig 3A. Neither diagnosis ($P = .254$, Savage) nor disease status ($P = .847$, Savage) of the patients were significant.

Of the culture parameters examined, only the frequency of CFU-M in the recipient before administration of the preparative BMT regimen was significantly related to the time to engraftment ($P = .003$, Cox). Patients with higher CFU-M values before transplantation engrafted significantly faster than did patients with low CFU-M values. The data are displayed as Kaplan-Meier plots comparing the subgroup of patients that formed more than the median number of megakaryocyte colonies in comparison to patients with a colony frequency below the median (Fig 3B). The correlation between CFU-M and time to engraftment remained significant even if patients with ALL who represented a subpopulation with higher CFU-M values were excluded from the analysis ($P = .028$, Savage).

The concentration of CFU-M in recipient marrow before transplantation also predicted the reemergence of platelets and reticulocytes when assessed individually. The frequency of CFU-GEMM and BFU-E was correlated with the time to

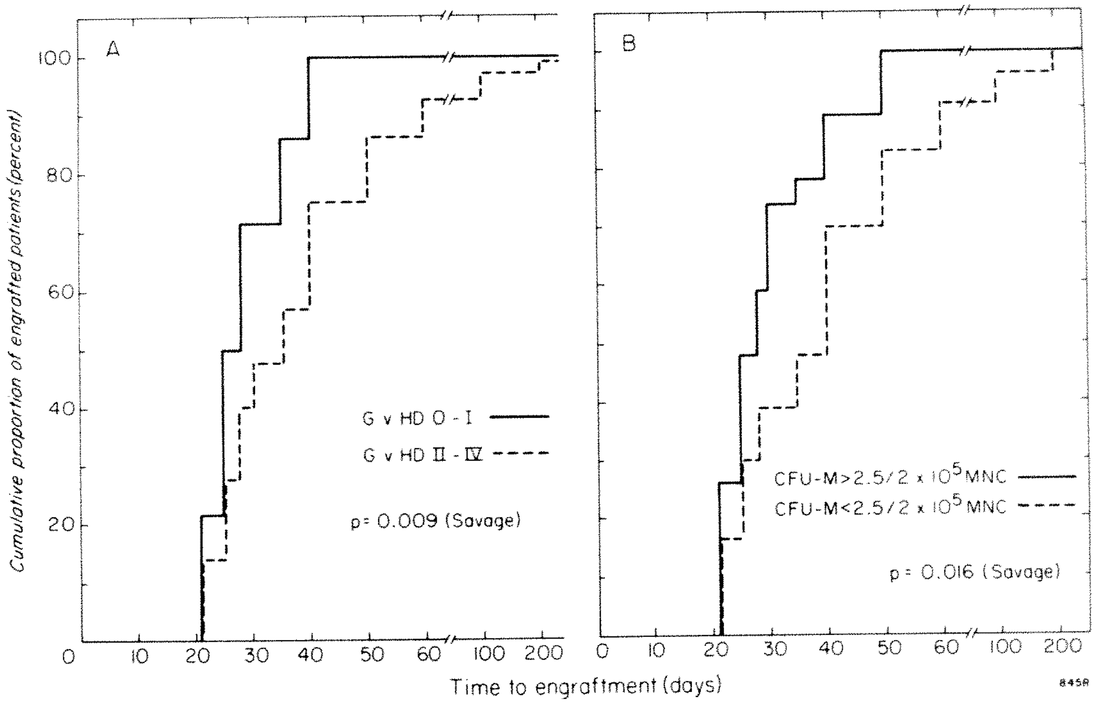

Fig 3. **(A) Time to engraftment for patients with GvHD grades 0 to I and II to IV. (B) Time to engraftment for recipients who formed <2.5 or >2.5 megakaryocyte colonies for 2 × 10^5 mononuclear cells in bone marrow samples obtained before transplantation.**

recovery of reticulocytes ($P = .023$ for CFU-GEMM, and $P = .012$ for BFU-E, Cox), but not of other lineages. The data were further analyzed using multi-predictor Cox regression models to determine whether there was a significant interaction between CFU-M and GvHD and to test whether the inclusion of clinical parameters in the model negated the influence of CFU-M.

First, a regression model including age, sex, and weight of the recipient, pre-BMT frequency of CFU-M, occurrence of GvHD, and an interaction between CFU-M and GvHD was examined. The influence of CFU-M ($P = .002$) and GvHD ($P = .018$) on the time to engraftment was sustained independent of the clinical parameters. The interaction between CFU-M and GvHD was also significant ($P = .018$). The interaction indicated that the influence of CFU-M was greater for patients with GvHD.

Second, three regression models were constructed to test whether diagnosis or previous therapy, as measured by disease status, affected the significance of either CFU-M or GvHD. These models included the same parameters as above but, in addition, were stratified by (a) diagnosis, (b) disease status, and (c) both. In each of the three models CFU-M ($P = .0015$ to $.0069$) and GvHD ($P = .013$ to $.02$) remained strongly significant. The multipredictor Cox regression results indicate that both CFU-M and GvHD are significantly related to time to engraftment, independent of the other clinical parameters tested.

Analysis of parameters influencing survival time. Similar studies were performed to assess the influence of the same cell culture and clinical parameters on survival time (Table 4). Again all parameters were initially assessed alone and subsequently in conjunction with each other using the Cox regression model.

The univariate analyses showed that survival was significantly influenced by the disease status at the time of transplantation. Patients with acute leukemia in first remission, chronic-phase CML, and aplastic anemia had a greater probability of surviving when compared with patients with more advanced disease ($P = .007$, Savage). The data are displayed as Kaplan-Meier plots in Fig 1B. No other clinical parameter was significant.

Of the culture parameters, the frequency of clonogenic precursors in recipients prior to transplantation and number of clonogenic progenitors in each transplant inoculum were not predictive of survival. In contrast, the total number of nucleated cells in the transplant inoculum made a significant contribution ($P = .042$, Cox). Patients transplanted with a larger inoculum had better overall survival than did patients who had received a lower cell load. For the purpose of a visual display as Kaplan-Meier plot, patients were subdivided into a group below and above the median number of transplanted nucleated cells (Fig 1C). A correlation with survival was not observed when the number of nucleated cells transplanted per kilogram of the recipient's body weight was used for the analysis ($P = .185$, Cox).

A multivariate analysis was performed to test the influence of the total number of nucleated cells in conjunction with other parameters. The Cox regression model included age, sex, weight, and was stratified by risk group as defined by disease status. The influence of the total number of nucleated cells in the transplant inoculum on survival was sustained ($P = .042$) independent of the contribution of the other parameters.

DISCUSSION

The reestablishment of hemopoiesis after BMT is a multifactorial process that involves a number of donor and host parameters. We examined clonogenic hematopoietic precursors in the host before and serially after BMT and determined their quantity in donor-derived BMT inocula. The frequency of clonogenic progenitors in bone marrow samples collected from most BMT recipients before administration of the ablative pre-BMT regimen was significantly lower than that observed for the respective donor values. The resulting frequency distributions were skewed toward low levels. Only BMT recipients with ALL had normal progenitor numbers and distribution profiles.

Colony formation after transplantation did not return to the level of their respective donors but approximated the low pre-BMT values. The data obtained post-BMT for patients with ALL were indistinguishable from results of recipients with other primary diseases. These data suggest that hematopoiesis after transplantation is maintained by a reduced number of clones. This view is supported by animal experiments using retrovirus-transduced stem cells for reconstitution of lethally irradiated mice. Lemischka et al[14] showed that animals were repopulated by clones derived from as few as 1 or 2 stem cells. The increased proportion of pluripotent precursors in S-phase therefore reflects attempts to compensate for a decreased bone marrow reserve.

Our data describing a reduced frequency of pluripotent and committed precursors confirm data previously reported for CFU-GM and BFU-E by Arnold et al[1] and CFU-GM by Li et al.[2] They differ from observations by Norrie et al,[15] indicating return to normal values of CFU-GM, CFU-E and BFU-E in their series of 29 patients. The reason for this difference is not clear. It may relate to differences in the culture technique, expression of the data as number per volume of bone marrow or blood rather than as plating efficiency. Differences in the preparative regimen may affect the regenerative capacity of the bone marrow by influencing for instance the stromal support.

In addition, as suggested by Li et al,[2] one may postulate the suppression of clonogenic precursors after BMT by cellular or molecular mechanisms. We removed E rosette-positive cells from four bone marrow samples obtained after BMT and observed a significantly greater than anticipated increment in the plating efficiency of one specimen.

The observation that the number of nucleated cells may influence survival of the examined patient population remains of practical interest since it is technically feasible to increase the number of transplanted cells. The importance of the size of the BMT inoculum for sustained and stable engraftment and reestablishment of a normal reserve has been well documented in murine hemopoiesis. Serial retransplant experiments demonstrated that only bone marrow of primary transplant recipients who had received a large marrow inoculum was able to repopulate secondary hosts.[16,17]

The importance of cell number in clinical transplantation was previously demonstrated for aplastic anemia by Storb et al,[18] who reported in an analysis of 73 patients a higher rate of graft rejection for patients transplanted with 3 × 10^8 nucleated cells/kg. In addition, Ringden and Nilsson[19] demonstrated that patients with GvHD had a significantly better chance of surviving if they were transplanted with a higher total cell dose. Future clinical studies are necessary to determine whether a larger BMT inoculum will improve the bone marrow reserve in BMT recipients.

Investigators have addressed the question of whether time to engraftment is related to the size and cellular composition of the transplant inoculum. Niederwieser et al[20] showed for 50 patients with acute or chronic myeloid leukemia that a negative nonlinear correlation existed between the number of transfused cells and the duration of leukopenia. Faille et al[21] demonstrated a correlation between the number of CFU-GM and the time to recovery of neutrophils for ten patients with aplastic anemia. A similar observation was made by Spitzer et al[22] for hematopoietic recovery following autologous BMT. In contrast, Torres et al[23] were unable to identify a relationship between the number of transplanted CFU-GM and recovery of peripheral granulocytes in a series of 16 patients transplanted for acute leukemia. Similar results with respect to the total cell number, CFU-GM and CFU-E dose were observed by Atkinson et al.[24]

In our own study, time to engraftment was not correlated with any one of the cell populations in the transplant inoculum. We observed, however, that time to engraftment was significantly influenced by two host-related parameters. First, the development of GvHD, or possibly the therapy for GvHD, played a significant role in the delay of engraftment. Second, the frequency of CFU-M in the recipient before administration of their preparative regimen reflected the speed of recovery of all hematopoietic lineages. This correlation is upheld independent of diagnosis or disease status at the time of transplantation.

It was surprising that CFU-M in the host before BMT reflected the time of bone marrow recovery more accurately than did the pluripotent precursor CFU-GEMM. The reason for this correlation is not currently understood. CFU-M may reflect the behavior of a more primitive progenitor cell population that is essential for engraftment. This view is supported by a report that CFU-M in murine hematopoiesis and day 14 CFU-S bear similar surface determinants detected by the monoclonal antibody denoted as Qa-M^2,[25] whereas the antigen is not expressed by day 7 CFU-S considered to be more mature. Alternatively, CFU-M may represent a precursor population that requires optimal growth support in vivo. Their frequency may reflect the functional status of the growth-supporting hematopoietic microenvironment of the host and its ability to promote the proliferation of transplanted precursors. This hypothesis is consistent with the observation that BMT recipients usually establish erythropoiesis and granulopoiesis before platelets are produced at an adequate rate.

Examination of the role of CFU-M in the host is necessary. A preliminary study has indicated that the host may produce stimulatory molecules for CFU-M and, to a lesser degree, for other clonogenic precursors shortly after receiving the preparative regimen.[26] These can be readily identified in the plasma of transplant recipients and may serve as an additional measurement to examine hematopoietic regeneration after transplantation.

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High-Dose Intravenous Gamma Globulin Improves Responses to Single-Donor Platelets in Patients Refractory to Platelet Transfusion

By Zella R. Zeigler, Richard K. Shadduck, Craig S. Rosenfeld, Kenneth F. Mangan, Alan Winkelstein, Arsinur Oral, Glenn E. Ramsey, and Rene J. Duquesnoy

Ten patients, with bone marrow failure or malignant disorders, became refractory to platelet transfusions using random, as well as partial or fully HLA-matched, single-donor platelets. To determine its effect on platelet refractoriness, intravenous gamma globulin (IV IgG) was administered at 400 or 800 mg/kg/d for five days, and postinfusion platelet responses were monitored. Platelet transfusion responses following intravenous gamma globulin (IV IgG) were graded as follows: Excellent, 48-hour posttransfusion count > 50,000/μL; good, 48-hour count > 20,000 but <50,000/μL; Fair, increased increment, 48-hour count < 20,000; and failed, no increased increment. Six of ten patients (60%) had improved responses to selected single-donor platelets (two were excellent, three were good, and one was fair). The time to achieve a platelet transfusion count >25,000/μL ranged from one to nine days of IgG therapy. One individual had sustained benefit (>1 year); the remaining responses persisted for 6 to 8 weeks. These results suggest that IV IgG may be useful in the management of platelet refractoriness, especially in patients receiving single-donor platelets.

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PLATELET refractoriness represents a life-threatening complication in patients with bone marrow failure who have platelet counts <10,000/μL. Poor platelet increments occur in two situations and may have different pathogenic mechanisms. In outpatients with chronic aplasia, poor responses probably reflect alloimmunization.[1-3] In contrast, in febrile patients following chemotherapy-induced aplasia, factors related to infections may contribute to poor transfusion responses. In these patients, it is difficult to discern the relative contribution of allosensitization and/or other factors.

High-dose intravenous gamma globulin (IV IgG) is effective in the treatment of autoimmune thrombocytopenic purpura[4-6] and posttransfusion purpura.[7-10] Results are conflicting, however, in the management of platelet alloimmunization.[11-17] IV IgG is potentially suitable for the management of platelet refractoriness because it has a rapid onset of effect (two to five days) and does not suppress bone marrow.[4-6] We report the beneficial effects of IV IgG in the management of a cohort of platelet-refractory patients.

MATERIALS AND METHODS

Patients. Ten patients who became refractory to platelet transfusion, defined as a one-hour posttransfusion increment of <15,000/μL, participated in this study. All ten had failed to respond to 24 to 110 U random-donor platelets, (administered as 6- to 10-U platelet transfusions) and to platelet transfusions from donors who were HLA-identical, partially matched, or selectively mismatched, for cross-reactive HLA antigens.[18-20] Each patient gave informed consent for participation in this study. The protocol was approved by the Institutional Review Board of Montefiore Hospital, University of Pittsburgh School of Medicine.

The clinical characteristics of the patients are shown in Table 1; four patients (patients no. 1 through 4) had chronic aplastic anemia and became refractory to prophylactic transfusion of selected single-donor platelets. Patient no. 4 developed pneumonia five days after initiation of IV IgG therapy; the other patients with aplasia were afebrile. Three patients (patients 5 through 7) had bone marrow failure due to lymphoma and developed refractoriness to chronic platelet transfusion therapy; one (patient no. 7) was receiving active chemotherapy. The remaining patients failed to obtain platelet responses to transfusion while undergoing induction therapy for acute nonlymphocytic leukemia (ANLL).

Patients varied in the duration of blood support (2 weeks to 3 years) and the number of unit exposures (13 to 735 U). Generalized alloimmunization, as judged by HLA-antibody screening, was detected in eight patients; these patients showed antibodies against >75% of screening cells. Six had fever, two had enlarged spleens, and 1 (patient no. 5) had undergone splenectomy earlier in her treatment course. HLA-identical platelets were available and ineffective in six of the ten patients. Five had gastrointestinal (GI) bleeding (melena, hematemesis, or rectal bleeding), and one suffered limited hemorrhage into the CNS during the time of study. Three patients (patients no. 2, 5, and 6) who failed to respond died of uncontrolled bleeding.

IgG infusion. All patients received IgG (Sandoglobulin) as an IV infusion over six to eight hours at a dose of 400 mg/kg/d for five days. This dose was chosen to correspond to that recommended for the treatment of immune thrombocytopenia purpura. If no response occurred during the five-day course of therapy, the dosage was increased to 800 mg/kg/d until a posttransfusion platelet count ≥25,000/μL was obtained or for an additional five days. Single-donor platelets were given within one to ten hours of the IgG infusion if the platelet counts were <20,000/μL. Random-donor platelets were not given unless the patient was actively bleeding and single-donor platelets were ineffective.

Definition of platelet responses. One-hour posttransfusion platelet counts were performed by chamber analysis using the method of Brecher and Cronkite.[21]

Responses to single-donor platelets were graded as follows: Excellent, increased increment, 48-hour count >50,000/μL; good, increased increment, 48-hour count > 20,000 but < 50,000/μL; fair, increased increment, 48-hour count <20,000/μL; and failed, no increased increment.

For single donors that were used repetitively for a given patient, the degree of HLA match to the patient was determined. This was based on HLA-A and HLA-B loci and published cross-reactivity information.[18-20] The match categories were defined as follows: A

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Table 1. Platelet Refractory Patients: Clinical Characteristics

Sex	Age	Diagnosis	Blood Support (mos)	Blood or Platelets Administered (U)	HLA Screen No. Positive/ No. Screening Cells	Temperature $>100°F$	Spleen	A-match Donor
M	14	Chronic aplastic anemia	5.0	735	+ 28/30	N	NP	NA
M	57	Chronic aplastic anemia	13.0	149	+ 40/48	N	NP	NA
M	59	Chronic aplastic anemia	1.0	115	+ 29/30	N	NP	Failed
F	56	Chronic aplastic anemia	5.0	191	+ 17/20	Y	NP	NA
F	62	Lymphoma	17.0	315	+ 21/48	N	Splenex	Failed
F	53	Lymphoma	15.0	477	+ 21/24	Y	Enlarged 6 cm	Failed
F	60	Lymphoma	1.0	59	+ 36/48	Y	Enlarged 3 cm	Failed
M	24	Acute aplasia ANLL	3.5	156	+ 36/48	Y	NP	Failed
F	60	Acute aplasia ANLL	1.0	174	+ 27/30	Y	NP	Failed
F	77	Acute aplasia ANLL	0.5	13	+ 10/48	Y	NP	NA

N, no; Y, yes; NP, not palpable; Splenex, splenectomy; NA, not available.

match, HLA-A and HLA-B antigen identical; B match, three donor antigens matched and the fourth unknown or cross-reactive, or two donor antigens matched and the third and fourth unknown or cross-reactive; C match, three donor antigens matched and one mismatched; and D match, two or more donor antigens mismatched.

Platelet-associated IgG levels. Platelet-associated IgG levels were measured by the immunodiffusion assay of Morse et al.[22] Levels were monitored before and during the administration of IgG, and at times before the next transfusion, when the platelets were $<15,000/\mu L$. This was done so that changes would not be due to an increase in the platelet count.

RESULTS

Platelets from random donors were without benefit in six patients after administration of IV IgG. The results obtained with selected single donors are summarized in Table 2. The total amount of IV IgG administered varied from 2.0 to 6.0 g/kg. One patient (no. 4) continued to receive booster doses of 800 mg/kg before each platelet transfusion because of the fair response and the clinical judgment that continued IgG improved platelet increments. The other patients received only the initial course of IgG.

In this series, six patients had improved responses to platelet transfusion of selected single-donor platelets. Two of the responses were excellent, three were good, and one was fair. The time to a transfusion-related platelet count of $>25,000/\mu L$ ranged from one to nine days. In patients in whom the duration of response could be evaluated, most responses persisted from 6 to 8 weeks; however, one patient (patient no. 4) has had excellent responses to platelet transfusions in >1 year of observation.

The best response is summarized for patient no. 4 (Table 3), who had been supported by selected single donors for 5 months because of failure to respond to 30 U random-donor platelets. On three successive days, transfusion of platelets from donors that had been effective failed to provide adequate increments. The patient was admitted (October 10, 1984) for IV IgG in an attempt to improve platelet support. The first dose of IgG was administered, followed by platelet transfusion from donor no. 4, which provided a transfusion increment of $40,000/\mu L$, but a markedly shortened survival (the 24-hour count was $<16,000/\mu L$). Platelets from donor no. 4, on the seventh day of IV IgG, survived better, with a 48-hour count of $39,000/\mu L$. Improved increments are shown in Table 3 for each of the three donors used for this patient during the platelet refractory period.

Responses to platelets from single donors who were known to be effective before the refractory state were retested after IgG in three patients (patients no. 1, 4, and 8). The data from these seven donors is shown in Fig 1. For comparison, the 1-hour posttransfusion increment observed the first time a donor was used for that patient was considered to be 100%;

Table 2. Platelet Transfusion Responses After High-Dose IgG Therapy

Patient No.	Amount of IV IgG Administered (g/kg)	Response	Days to Platelets $\geq 25,000/\mu L$	Duration
1	6.0	Fair	7.0	8 weeks
2	6.0	Failed	—	Died of hemorrhage
3	6.0	Failed	—	Alive (platelets = $1,000/\mu L$)
4	2.0	Excellent	1.0	17 months
5	6.0	Failed	—	Died of hemorrhage
6	2.0*	Failed	—	Died of hemorrhage*
7	5.2	Good	9.0	>6 weeks†
8	2.0	Excellent	3.0	6 weeks‡
9	3.6	Good	5.0	>10 days* Died, refractory leukemia
10	6.0	Good	8.0	>11 days†

*Refused all further therapy.

†Bone marrow recovered.

‡Died of refractory leukemia at 8 weeks.

Table 3. Summary of Patient 4

Donor No.	Date	Platelet Count (μL) Pretransfusion	1-h Posttransfusion
A. Prerefractory period			
1	8/22/84	2,000	75,000
2	7/25/84	9,000	45,000
3	6/19/84	4,000	77,000
4	8/29/84	33,000	96,000
B. Refractory period			
1	10/8/84	5,000	21,000
2	10/9/84	4,000	16,000
3	10/10/84	3,000	15,000
C. Post IV IgG			
1	1/2/85	16,000	112,000
2	11/9/84	33,000	92,000
3	1/15/85	35,000	112,000
4	10/11/84	1,000	41,000
	(day 1 IV IgG)		(24-h < 16,000)
	10/17/84	7,000	70,000
	(day 7 IV IgG)		(48-h = 39,000)

the remaining results are expressed as a percentage of that baseline increment. Following IV IgG therapy, one-hour increments averaged 107.5% ± 16.7% (n = 16) of baseline as compared with 22.8% ± 2.8% (n = 8) in the refractory period of time, $P < .001$. The degree of HLA match between the donor and recipient did not appear to influence the results; improved increments occurred with grades of HLA match ranging from A through D.

Platelet associated-IgG (PA-IgG) levels were measured before transfusion and before and during therapy with IgG. Baseline values were elevated in seven of eight patients (Fig 2) and did not discriminate between responders and nonresponders. In contrast, a correlation was observed between responses and PA-IgG level obtained on days 7 through 15 of IV IgG therapy. In responders, values ranged from 3 to 11.4 fg/platelet; in nonresponders, the PA-IgG values ranged from 12 to 100. Responders showed a decline in PA-IgG; in contrast, nonresponders either showed no change or an increase in their levels.

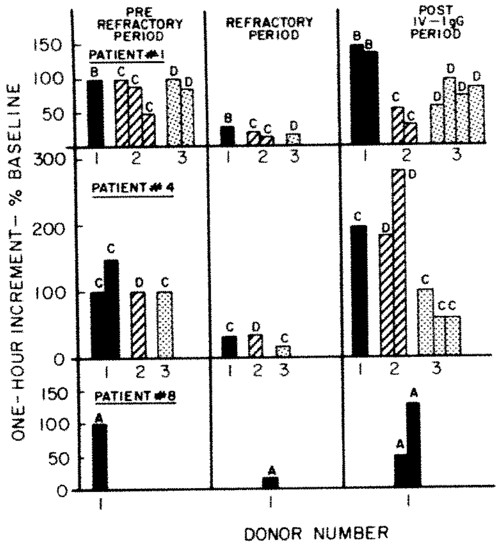

Fig 1. Comparison of one-hour posttransfusion platelet increments after use of selected single donors in prerefractory, refractory, and posttreatment periods in three patients. For comparison, all one-hour increments are expressed as percentage of baseline, (the increment obtained the first time that donor was used). (B through D): grade of HLA match of the donor.

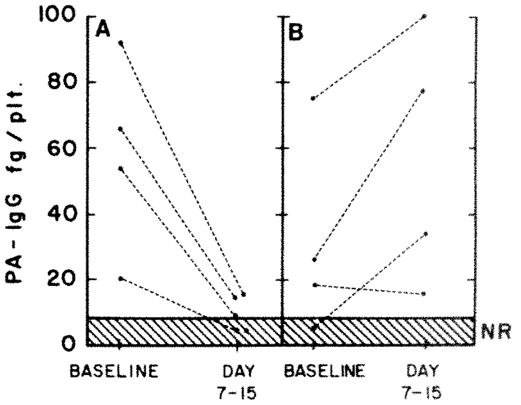

Fig 2. PA-IgG levels before and after IV-IgG treatment. PA-IgG levels were elevated in seven of eight patients prior to treatment (>6 fg/platelet). Repeat PA-IgG levels on days 7 through 15 of therapy were significantly lower in the group of responders as compared with nonresponders. This represented a decline of the levels in responders, whereas nonresponders either showed no change or an increase.

DISCUSSION

Although scattered reports[12-15,17] describe favorable responses to IV IgG in alloimmunized platelet transfusion recipients, the prevailing data are negative.[11,16] Studies by Schiffer et al[11] and Knupp et al[16] indicated no improvement in platelet transfusion responses to IV IgG. Thus, their results differ from those in this study.

From the present data, determination of the reasons for these discrepant results is impossible. Most of the patients reported herein received higher doses of IgG than were used in previous studies. Because the same preparation of IgG was used in both this study and that reported by Schiffer et al,[11] the choice of preparation does not appear to explain the difference. In this study, patients were given platelet transfusions in conjunction with the IgG therapy rather than having platelets administered after a course of IV IgG. Moreover, all the present patients were given the best HLA-matched single-donor platelets available. Further observations will be necessary to determine which, if any, of these differences are important in reversing the platelet-refractory state.

In this series, patients had improved responses to selected single-donor platelets; however, random-donor platelets were tried without benefit. Therefore, this therapy may not improve responses to random platelets in most cases, as has been noted previously.[11] That transfusion responses were restored to HLA-D–matched platelet products suggests that some responses to random products may be anticipated.

Although IV IgG improved responses to transfused platelets in selected patients, the mechanisms involved are unclear. In contrast to immune thrombocytopenic purpura in which data[5] suggest that platelet destruction is due to reticuloendothelial clearance of antibody-coated cells, the mechanism of destruction of allogeneic platelets is not established. Beneficial effects of IV IgG may also result from delayed clearance of sensitized cells. An analogy can be drawn to the findings of Grumet and Yankee,[2] who demonstrated that corticosteroids or splenectomy could improve transfusion increments to HLA- compatible, but not random-donor, platelets in alloimmunized aplastic patients.

The present observations suggest that IV IgG may be able to interfere with antibody coating of platelets. Decreased PA-IgG levels were observed as early as 1 day after institution of IgG therapy (data not shown). This decreased antibody binding continued throughout the study and correlated with response. PA-IgG levels on days 7 through 15 of therapy remained high in nonresponders and fell in responders. These results are similar to those of Becton et al,[13] who noted a decline in surface-associated IgG in two alloimmunized children with aplastic anemia that responded to IV IgG therapy. Moreover, Kekomaki et al,[12] showed that the addition of IgG, but not buffer, reduced alloantibody binding in vitro. Higher drug levels may be important for this mechanism as compared with those necessary to produce reticuloendothelial blockade.

In conclusion, high-dose IgG therapy improved platelet support, with use of single donors, in six of ten platelet-refractory patients. Even though the effect was usually transient (6 to 8 weeks), improved responses may provide valuable time needed for bone marrow recovery following therapy of acute leukemia or aplastic anemia. The use of IV IgG to improve responsiveness to transfused platelets is certainly expensive. In our hospital, 1 g of IV IgG costs \$100.00; thus, the average cost for a single course of 400 mg/kg for five days is ~\$14,000.00. Because of this, IV IgG is not a means of maintaining platelet counts $>20,000/\mu L$ prophylactically; however, this therapy might be considered if a patient is experiencing uncontrollable bleeding and is no longer supportable with single-donor platelet products.

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Fibronectin Is Required for Platelet Adhesion and for Thrombus Formation on Subendothelium and Collagen Surfaces

By Eva Bastida, Ginés Escolar, Antonio Ordinas, and Jan J. Sixma

Fibronectin (FN) plays a role in several adhesion mediated functions including the interaction of platelets with subendothelium. We investigated the role of plasma FN in platelet adhesion and platelet thrombus formation under flow conditions. We used two different perfusion models: the annular chamber with α-chymotrypsin–treated rabbit vessel segments, and the flat chamber with coverslips coated with fibrillar purified human collagen type III. Perfusates consisted of washed platelets and washed RBCs, suspended in normal or FN-depleted plasma. Perfusions were carried out for ten minutes at shear rates of 300 or 1,300 s^{-1}. Platelet deposition and thrombus dimensions were evaluated morphometrically by a computerized system. We found that depletion of plasma fibronectin significantly reduced the percentage of total coverage surface and percentage of platelet thrombus, at both shear rates studied, and in both perfusion systems ($P < .01$) ($P < .01$). The dimensions of the platelet thrombi formed in perfusions at high shear rate were also significantly reduced in perfusions carried out with FN depleted plasma ($P < .01$). Addition of purified FN to FN-depleted perfusates restored all values to those measured in the control perfusions. These results indicate that plasma FN is required for platelet aggregate and thrombus formation following adhesion under flow conditions.

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PLATELET ATTACHMENT, spreading and aggregation on vascular subendothelium are crucial events in platelet thrombus formation. Several factors are known to regulate these events: physical factors,[1,2] subendothelial components,[3] adhesive proteins,[4] and specific receptors on the platelet membrane.[5]

Fibronectin (FN) is a multifunctional glycoprotein with numerous biological roles. Besides its interactions with platelets, FN is known to promote attachment and spreading of cells on collagenous substrates,[6] and to bind to different types of cells such as fibroblasts,[7] rat kidney cells,[8] and malignant melanoma cells.[9] Recent studies have demonstrated that the Arg-Gly-Asp sequence within FN is involved in the cell attachment function of this glycoprotein. It has been hypothesized that platelets have a receptor capable of recognizing the Arg-Gly-Asp sequence in several adhesive proteins and that this platelet receptor is the membrane glycoprotein GPIIb/IIIa.[10] This amino acid sequence has also been found in other proteins that interact with platelets such as fibrinogen, von Willebrand factor, and vitronectin.[11] On the other hand, it has been reported that platelets from thrombasthenic patients lacking the GPIIb/IIIa complex, do not form thrombi in the Baumgartner perfusion system, using both citrated[12] and nonanticoagulated blood.[13]

In this study, we investigated the role of FN in platelet adhesion and in platelet thrombus formation. We used two perfusion models: the annular chamber with rabbit abdominal aorta segments and the flat chamber with coverslips coated with fibrillar purified human collagen type III. Morphometry was used to measure the extent and the state of interacting platelets, as well as to determine platelet thrombus dimensions.

MATERIALS AND METHODS

Collagens

Collagen type III from human umbilical arteries was isolated and purified by the Chandrajajan method.[14] Collagen fibers were prepared as previously described.[15] Briefly, native type fibers were prepared by dissolving the collagen (1 mg/mL) in 50 mmol/L acetic acid and dialized against 20 mmol/L Na_2HPO_4 at 4°C for 48 hours. The collagen fibrils were collected by centrifugation (30 minutes, 30,000 g, 4°C) and resuspended in 20 mmol/L Na_2HPO_4. The collagen concentration in the fibrillar suspension was determined by the hydroxyproline assay.

Fibronectin Depleted Plasma and Fibronectin

FN was isolated from plasma by affinity chromatography as described.[16] FN-depleted plasma contained no detectable FN on electro-immunoassay. The level of FVIIIR:Ag was unchanged and the level of FVIII:RCo ranged from 0.7 to 0.85 U/mL. There was essentially no change in the multimeric structure of von Willebrand factor after passage over the gelatin column. Purity of the isolated FN was assessed by SDS gel electrophoresis according to Laemmli.[17]

Preparation of Perfusates

Blood from normal healthy donors who had taken no drugs ten days before blood collection was anticoagulated with 1:10 vol 110 mmol/L trisodium citrate. Platelet rich-plasma was prepared by centrifugation (ten minutes, 150 g, 20°C). Platelets were washed twice by centrifugation (ten minutes, 500 g, 20°C) in Krebs-Ringer buffer pH 6.0. RBCs were washed three times by centrifugation (ten minutes, 300 g, 20°C) in isotonic saline containing 5 mmol/L glucose. Perfusates were prepared as previously described[15] and consisted of washed platelets (WP), washed RBCs (WRBC) and normal plasma or FN-depleted plasma. Before each perfusion run, the perfusate (15 mL) was reconstituted by adding WRBC to the resuspended platelets (hematocrit 0.4, platelet count 1.9×10^{11} pl/L, final concentration). In some experiments, FN was added to

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Table 1. Platelet Deposition on α-Chymotrypsin-Treated Rabbit Vessels

Perfusate	Vessel Surface Covered (%)				
	Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma	3.6 ± 1.0	6.8 ± 0.1	3.6 ± 0.3	3.5 ± 0.5	17.6 ± 0.5
FN-depleted plasma	2.7 ± 0.1	4.0 ± 0.3	1.5 ± 0.3*	1.5 ± 0.3*	9.8 ± 0.4*
FN-depleted plasma + FN	4.0 ± 2.0	6.6 ± 0.2	3.2 ± 0.9	3.0 ± 1.0	16.8 ± 0.9

α-Chymotrypsin-digested rabbit vessels exposed to blood for ten minutes at a shear rate of 300 s^{-1}. Values represent mean ± SEM (n = 5).

*$P < .01$.

FN-depleted plasma to obtain a final concentration of 300 μg/mL. The perfusates were incubated for five minutes in a water bath at 37°C before starting the perfusion.

Preparation of the Vessels

Rabbit abdominal aorta segments were obtained from New Zealand rabbits that weighed 2.5 to 2.8 kg. Arteries were excised above the iliac artery and were everted to expose the luminal surface. The endothelium was removed by treatment with α-chymotrypsin as previously described.[18] The everted, de-endothelialized segments, ~14 mm in length, were stored at −80°C until used. Before perfusion, vessel segments were rinsed with 0.02 mol/L phosphate buffered saline (PBS) pH 7.4.

In some experiments, α-chymotrypsin-digested rabbit segments were incubated with purified FN at a concentration of 1 mg/mL for 30 minutes at 20°C. The preincubated vessel segments were thoroughly rinsed with PBS pH 7.4 before being used in perfusions.

Preparation of the Coverslips

Fibrillar collagen (1 mg/mL) was sprayed onto plastic coverslips (Thermanox, Miles Laboratories, Naperville, IL) at a concentration of 20 μg/cm² with a retouching airbrush, as previously described.[15]

Perfusion Experiments

Perfusions were carried out in annular chambers according to Baumgartner,[19] and in flat chambers as described by Sakariassen et al.[20] The annular chambers were used for the arteries and the flat chambers were used for collagen-coated coverslips.

Processing of Vessel Segments and Plastic Coverslips for Cross-Sectional Morphometric Evaluation

Vessel segments. After perfusion, the segments were rinsed with PBS and fixed with glutaraldehyde. The fixed segments were sliced from the supporting rod, dehydrated through a graded series of ethanol concentrations, embedded in JB-4 embedding media (KIT composed of components A, B, and C Polysciences, Warrington, PA), thin-sectioned for light microscopy, and stained with toluidine blue as described.[21]

Collagen-coated coverslips. In perfusions carried out with the flat chambers, the system was rinsed with PBS pH 7.4. following the experiments. The perfused coverslips were carefully removed from the chamber knobs, rinsed thoroughly with 0.02 mol/L PBS pH 7.4 and fixed for one hour in 0.5% glutaraldehyde in 0.1 mmol/L PBS pH 7.4. After one hour of fixation, coverslips were washed in fresh PBS and dehydrated in a graded series of ethanols (30%, 50%, 70%, 96%). Infiltration of the samples was performed in catalyzed JB-4 solution for two hours at 4°C. Embedding of the coverslips was performed in flat molds. The nonperfused side of the coverslips was laid on the bottom of the mold filled with catalyzed JB-4 solution. Polymerization was carried out at 4°C in special containers from which air had been removed by nitrogen current. After complete polymerization, plastic coverslips were carefully peeled off from the JB-4 polymer, leaving the perfused side of the coverslip firmly embedded in the JB-4 polymer. Three-millimeter wide strips of the JB-4 flat blocks were appropriately oriented and vertically embedded in BEEM capsules as described above. Final blocks were vertically sectioned with glass knives. Sections were removed from the cutting edge, floated on water, mounted on glass slides, dried, and stained with 1% methylene blue.

Morphometric Evaluation

Platelet interaction with subendothelium was evaluated according to the basic criteria described by Baumgartner[19] with minor modifications.[21] Basically, platelets or groups of platelets were classified as follows: contact (C), platelets that are attached but not spread on the subendothelium; spread (S), platelets that have spread on the subendothelial surface; aggregate (Ag), aggregates of <5 μm in height; thrombus (T), platelet aggregates of ≥5 μm in height. Total covered surface was obtained by adding C + S + Ag + T. Morphometric evaluation was performed using a manual optical picture analysis system (MOP 20 Kontron) connected to a computer with an automated recognition program, as previously described.[21]

In addition to the morphometric evaluation, we simultaneously determined the cross-sectional areas of all platelet aggregates of >5 μm in height (Thrombus). The sum of all thrombus surface area expressed in square micrometers was related to the perimeter of either rabbit vessel or collagen coated coverslips, in each case 3,400 μm. The result obtained corresponded stereologically to the thrombus volume per unit of surface area of the subendothelium (μm³/μm²), as described elsewhere.[22]

Table 2. Platelet Deposition on α-Chymotrypsin-Treated Rabbit Vessels

Perfusate	Vessel Surface Covered (%)				
	Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma (n = 7)	3.9 ± 0.6	10.8 ± 0.4	3.3 ± 0.5	14.4 ± 1.0	29.2 ± 3.7
FN-depleted plasma (n = 6)	3.9 ± 0.4	8.9 ± 1.7	1.6 ± 0.5	1.6 ± 0.1*	14.7 ± 3.0*
FN-depleted plasma + FN (n = 3)	4.1 ± 0.9	13.2 ± 2.8	6.5 ± 2.2	8.6 ± 1.8	32.4 ± 6.3

α-Chymotrypsin-digested rabbit vessels exposed to blood for ten minutes at a shear rate of 1,300 s^{1}. Values represent mean ± SEM with the number of experiments in parentheses.

*$P < .01$.

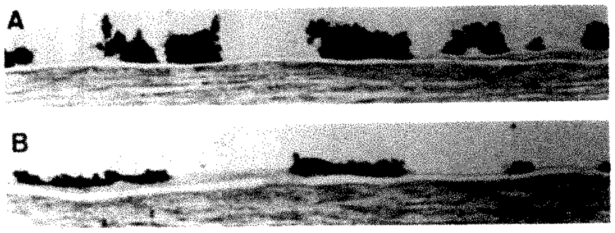

Fig 1. Light micrographs (original magnification ×1,000; current magnification ×590) of α-chymotrypsin treated rabbit subendothelium after ten minute perfusions at a shear rate of 1,300 s^{-1}. (A) Normal plasma. (B) FN-depleted plasma.

RESULTS

Studies on Platelet Deposition on Rabbit Subendothelium

To assess the role of plasma FN in the interaction of platelets with subendothelium of different thrombogenic properties, we carried out perfusions with vessel segments that had been de-endothelialized by overnight digestion with α-chymotrypsin. Perfusions were performed at two different shear rates, 300 and 1,300 s^{-1}, respectively. Table 1 summarizes the results obtained in the perfusions at a shear rate of 300 s^{-1}. In this group of experiments, plasma FN depletion significantly reduced the percentage of platelet thrombi from 3.5% ± 0.5% to 1.5% ± 0.3% ($P < .01$) and the total coverage surface 17.6% ± 0.5% to 9.8% ± 0.4% ($P < .01$). The percentage of the surface covered by contact and spread platelets, and the platelet aggregates of <5 μm in height were also reduced, although these values were not significant.

Perfusions carried out with α-chymotrypsin–digested rabbit vessels at a high shear rate (1,300 s^{-1}) indicated the role of plasma FN in platelet thrombus formation (Table 2). In these experiments, the surface coverage with platelet thrombi decreased from 14.4% ± 1.0% to 1.6% ± 0.1% ($P < .01$). The total surface coverage was also significantly diminished from 29.2% ± 3.7% to 14.7% ± 3.0% ($P < .01$). No significant differences were observed for the values of contact and spread platelets. Surface coverage with aggregates of <5 μm in height was slightly decreased but of no statistical significance. Figure 1 illustrates the differences by showing micrographs of the vessel wall subendothelium after perfusions at a shear rate of 1,300 s^{-1} with perfusates prepared with normal plasma (Fig 1A) or FN-depleted plasma (Fig 1B).

In both groups of experiments, at low and high shear rates, addition of 300 μg/mL of FN to FN-depleted perfusates restored the values of platelet deposition to those measured in control perfusions.

Treatment of the rabbit vessel with α-chymotrypsin for 18 hours digests all noncollagenous subendothelial proteins, including FN. Attempts to restore vessel FN were made by preincubating the digested vessels with purified human FN. Preincubation of enzymatically-treated rabbit vessel segments with purified human FN (1 mg/mL) for 30 minutes at 22°C did not modify the pattern of platelet adhesion. These results are summarized in Table 3. No significant changes were observed after preincubation of the vascular material with purified FN. For these experiments, FN-depleted plasma was used in the perfusate.

Platelet Deposition on Nonfibrillar and Fibrillar Purified Collagen Type III

To study the role of plasma FN on platelet adhesion to human purified collagen type III, perfusions using the flat chamber perfusion system were performed.[20]

Results are summarized in Table 4. At a shear rate of 300 s^{-1}, the vessel surface covered with platelet aggregates of <5 μm, with platelet thrombi, and the total coverage surface, were reduced from 14.2% ± 1.7% to 8.3% ± 0.8%, from 6.1% ± 1.7% to 2.0% ± 0.7%, and from 31.0% ± 5.0% to 14.8% ± 3.7%, respectively in perfusions carried out with FN-depleted plasma. All these values reached significant levels ($P < .01$).

The values obtained in perfusions performed at a shear rate of 1,300 s^{-1} also indicated the role of plasma FN in the interaction of platelets with purified fibrillar collagen type III. The most significant reduction observed in perfusates prepared with FN-depleted plasma was in the percentage of platelet thrombi, with a decrease from 14.3% ± 3.0% to 3.6% ± 1.0% ($P < .05$). The values of platelet aggregates and of the total coverage surface were also decreased, but were of no significance.

Measure of Platelet Thrombus Volume

The dimensions of the thrombi formed in perfusions carried out at high shear rate with normal, FN-depleted plasma, and FN-depleted plasma to which FN had been added, were performed using the computerized system for morphometric evaluation. The results are shown in Fig 2. In perfusions performed with the annular chamber at a shear rate of 1,300 s^{-1}, the mean thrombus volume per surface area was 0.58 ± 0.11 $\mu m^3/\mu m^2$ for normal perfusates, and 0.12 ± 0.08 $\mu m^3/\mu m^2$ for FN-depleted perfusates, respectively.

Table 3. Platelet Deposition on α-Chymotrypsin–Treated Rabbit Vessels Preincubated With Purified FN

Vessel Wall Preincubation	Shear Rate (s^{-1})	Vessel Surface Covered (%) Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Buffer (n = 4)	300	2.9 ± 0.6	4.3 ± 0.9	2.0 ± 0.4	1.8 ± 0.5	11.0 ± 1.9
FN (n = 4)	300	3.1 ± 0.7	3.8 ± 1.0	2.3 ± 0.5	1.6 ± 0.4	10.8 ± 2.4
Buffer (n = 4)	1,300	3.9 ± 0.4	10.6 ± 2.5	3.0 ± 0.7	1.6 ± 0.1	17.2 ± 4.2
FN (n = 3)	1,300	4.1 ± 1.1	8.8 ± 2.2	2.1 ± 0.5	0.8 ± 0.4	15.0 ± 3.5

α-Chymotrypsin–digested rabbit vessels were preincubated with purified FN isolated from plasma by affinity chromatography and exposed to blood for ten minutes. Perfusates were prepared with FN-depleted plasma. Values represent mean ± SEM with the number of experiments in parentheses.

Table 4. Platelet Deposition on Fibrillar Collagen Type III

Perfusate	Shear Rate (s^{-1})	Surface Covered (%)				
		Contact	Spread	Ag < 5μm	Thrombus	Surface Coverage
Normal plasma (n = 4)	300	0.6 ± 0.2	10.1 ± 2.1	14.2 ± 1.7	6.1 ± 1.7	31.0 ± 5.0
FN-depleted plasma (n = 3)	300	0.4 ± 0.2	4.2 ± 2.5	8.3 ± 0.8*	2.0 ± 0.7*	14.8 ± 3.7*
Normal plasma (n = 3)	1,300	0.2 ± 0.05	8.1 ± 2.1	36.9 ± 5.3	14.3 ± 3.0	59.5 ± 10.4
FN-depleted plasma (n = 3)	1,300	0.6 ± 0.01	9.0 ± 1.8	29.8 ± 3.5	3.6 ± 1.0*	43.0 ± 6.7

Fibrillar collagen type III was exposed to blood for ten minutes. Values represent mean ± SEM with the number of experiments in parentheses.
*$P < .01$.

These values reached the significant level ($P < .01$). Addition of purified FN to FN-depleted perfusates restored the value of thrombus volume to 0.48 ± 0.12 $\mu m^3/\mu m^2$.

Measure of thrombus volume in perfusions carried out with the flat chamber, using human fibrillar collagen as the substrate for platelet adhesion, displayed similar results. In this set of experiments, a statistically significant decrease ($P < .01$) in the mean thrombus volume per surface area was 0.67 ± 0.09 $\mu m^3/\mu m^2$ in normal perfusions and 0.20 ± 0.10 $\mu m^3/\mu m^2$ in FN-depleted perfusions was observed. The results obtained following the addition of 300 μg/mL of purified FN to FN-depleted perfusates showed a value of 0.60 ± 0.15 $\mu m^3/\mu m^2$, which was similar to the value measured in control perfusions. These results, using both perfusion systems, indicate the importance of plasma FN in platelet thrombus formation.

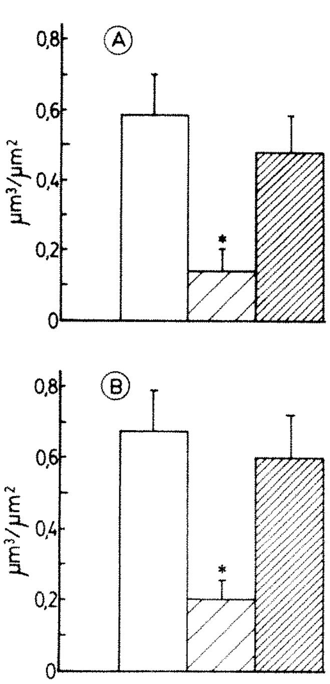

Fig 2. Effects of plasma FN on platelet thrombus formation Platelet thrombus volume per surface area was measured in ten minute perfusions at 1,300 s^{-1} and expressed in $\mu m^3/\mu m^2$. (A) Perfusions were performed with the annular chamber using α-chymotrypsin–digested rabbit segments. The number of experiments were n = 7, n = 6, and n = 3 for normal plasma, FN-depleted plasma, and FN-depleted plasma + FN, respectively. (B) Perfusions were performed with the flat chamber using fibrillar human collagen type III. The number of experiments were n = 4, n = 3, and n = 4 for normal plasma, FN-depleted plasma, and FN-depleted plasma + FN, respectively. □, Normal plasma; ▨, FN-depleted plasma; ▨, FN-depleted plasma + FN. Bars indicate mean thrombus volume per surface area and lines indicate SEM. $P < .01$.

DISCUSSION

It is well documented that FN plays a role in some adhesion-mediated cell functions, including the interaction of platelets with vessel subendothelium.[15,23] In the present study, we investigated the role of plasma FN in platelet adhesion and platelet thrombus formation under flow conditions. To expose flowing blood to collagenous subendothelial and purified human collagen type III surfaces two perfusion systems were used. Platelet-subendothelium interaction was studied using annular chambers, with α-chymotrypsin–digested rabbit arteries, and flat chambers, with coverslips coated with fibrillar human collagen type III. Our results indicated that plasma FN is required for platelet aggregate and platelet thrombus formation, following adhesion in both types of surfaces studied.

The experiments carried out with α-chymotrypsin–treated rabbit vessels, particularly those performed at high shear rate, in which the percentage of thrombus formed is higher, indicate that plasma FN plays an important role in thrombus formation. The fact that average thrombus volume was significantly reduced in FN-depleted perfusions and that the addition of purified FN to the perfusates restored the values to those measured in control perfusions, strongly supports that plasma FN is not only important for the adhesion of platelets to subendothelium, but also for subsequent platelet thrombus growth under flow conditions.

Our results do not agree with those of Houdijk and Sixma,[23] who reported that FN in human artery subendothelium, but not in plasma, was important for platelet adhesion. However, more recently the same investigators found that platelet adhesion to endothelial cell matrix presented a shear-rate dependent requirement for plasma FN.[4] These differences in the relative role of plasma FN on platelet adhesion can probably be explained by differences between the surfaces used for platelet adhesion studies. In our studies, we used α-chymotrypsin–digested rabbit vessels that, as assessed by immunofluorescence staining (unpublished observation), were found to lack intact subendothelial FN, following enzymatic treatment. Preincubation of digested vessels with purified human FN did not enhance platelet deposition on subendothelium at any shear rate studied. This lack of effect may be due to human FN not incorporating into the collagen network of the digested rabbit vessels. This may be caused by differences between animal species or by steric hindrance due to remaining FN fragments.

The experiments performed with the flat chamber using fibrillar collagen type III as a substrate for platelet adhesion displayed similar results to those obtained with enzymatically digested vessel subendothelium. In this system, plasma FN was also found to be required for platelet adhesion and platelet thrombus formation on collagen coated surfaces, at both low and high shear rates. The observation that in both experimental systems used, addition of purified FN to the perfusates restored the values obtained for control perfusions indicating that the effects on platelet deposition were due to depletion of plasma FN.

Until now other plasma proteins such as fibrinogen[24] and von Willebrand factor[25] have been suggested to play a role in platelet-platelet interaction. There is evidence that fibrinogen, von Willebrand factor, and FN bind to the GPIIb/IIIa membrane complex of activated platelets. Recently, various investigators explored the role of GPIIb/IIIa in platelet adhesion to subendothelium, using the Baumgartner perfusion system. Sakariassen et al[12] showed a defect in platelet adhesion at high shear rates after blocking the GPIIb/IIIa complex with specific monoclonal antibodies. Furthermore, Weiss et al[13] reported that platelets deficient in GPIIb/IIIa do not spread normally on the subendothelial surface. However, the specific binding site of FN to the GPIIb/IIIa receptor and its implications in platelet function are still not well understood. Thus, it has been reported that FN binds to specific divalent cation-dependent receptors on washed, thrombin stimulated platelets,[26] but does not bind to ADP- or epinephrine-stimulated platelets, whereas, fibrinogen and von Willebrand factor do.[27] These observations suggest separate binding sites for FN in the platelet membrane. Moreover, Dixit et al[28] described a monoclonal antibody directed against FN that does not interfere with the function of the cell binding domain of the FN. The ability of this antibody to inhibit platelet aggregation suggests that there is a functional site present in FN that has a direct role on platelet aggregation. Furthermore, the results reported by Parise and Phillips[29] demonstrate that FN can bind directly to purified GPIIb/IIIa complex, strongly suggesting that it has the potential to function as a platelet receptor for FN.

Our results indicated that under flow conditions plasma FN, in addition to supporting platelet adhesion to collagenous subendothelial surfaces and to purified collagen type III, influences platelet thrombus formation. It is feasible that binding of FN to GPIIb/IIIa, or to another putative receptor present in activated platelets, favors interaction between platelets and contributes to the development of platelet thrombi. In conclusion, the results we present here indicate that plasma FN is one of the adhesive proteins required for platelet thrombus growth under flow conditions.

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Membrane-Associated Sickle Hemoglobin: A Major Determinant of Sickle Erythrocyte Rigidity

By Evan A. Evans and Narla Mohandas

Micropipette aspiration tests on single erythrocytes have previously shown that the static rigidity (membrane shear modulus) of oxygenated sickle cells increased with increasing hemoglobin concentration, whereas the rigidity of normal cells was independent of hemoglobin concentration. Moreover, it was observed that after mechanical extension, sickle cells exhibited persistent deformation more frequently and to a greater extent than normal cells. To ascertain if differences in association of normal and sickle hemoglobin with the membrane could account for these observations, we measured rheologic properties of normal membranes reconstituted with sickle hemoglobin and sickle membranes reconstituted with normal hemoglobin. The static rigidity of normal ghosts reloaded with sickle hemoglobin was higher than those of either normal ghosts reloaded with normal hemoglobin or native normal cells. On the other hand, the increased rigidity of native sickle cells decreased to near-normal values following reconstitution with normal hemoglobin. Furthermore, we observed that normal ghosts reconstituted with sickle hemoglobin exhibited persistent bumps after mechanical extension, but no bumps formed on normal ghosts reconstituted with normal hemoglobin. Moreover residual bumps were not produced on sickle cells reloaded with normal hemoglobin. Since mechanical characteristics peculiar to sickle cells could be induced in normal cells by incorporation of sickle hemoglobin, *and* since normal characteristics could be restored to sickle cells by incorporation of normal hemoglobin, we suggest that the interaction of sickle hemoglobin with the cell membrane is responsible for augmented static rigidity of oxygenated sickle erythrocytes.

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STATIC and dynamic deformabilities of erythrocytes are important determinants of microcirculatory blood flow because they determine the rate of entry of erythrocytes into small capillaries, which in turn influences the pressure drop across the small vessels and the flow distribution within the microcirculation.[1-8] The microrheologic behavior of single erythrocytes is governed by both membrane and cytoplasmic properties. As such it has been difficult to evaluate whether or not sickle cell membrane deformability has been compromised or altered by the disease independent of the effect of sickle hemoglobin polymerization on the cytoplasmic viscosity. To determine whether or not the mechanical properties of the sickle cell membrane are altered, we previously studied static and dynamic deformability properties of normal and sickle erythrocytes in the oxygenated state as a function of cell hemoglobin concentration.[8] These studies showed that the dynamic rigidity was indistinguishable between oxygenated normal and sickle cells when examined as a function of hemoglobin concentration and that the dynamic rigidity was increased markedly with increasing cell hemoglobin concentration. On the other hand, the static rigidity of sickle cells was clearly greater than that of normal cells. Static rigidity of sickle erythrocytes increased with increasing hemoglobin concentration, reaching abnormally high values at cell hemoglobin concentrations above 46 g/dL, while static rigidity of normal erythrocytes was independent of hemoglobin concentration. A striking observation made during these studies was that sickle cells exhibited persistent or plastic deformation more frequently and to a greater extent than normal cells following the static membrane extension test. This type of material behavior indicated that there is probably a thin hemoglobin gel layer adsorbed to the cytoplasmic face of the membrane and that this layer is more developed in sickle cells than normal cells at equivalent cell hemoglobin concentrations.

To further illuminate the influence of membrane-associated hemoglobin on erythrocyte rheology, the following experimental approach was undertaken: sickle and normal erythrocytes were ghosted and reloaded with the opposite type of hemoglobin solution, and the membrane extensional properties were measured. We find that mechanical characteristics peculiar to sickle cells could be induced in normal cells by incorporation of sickle hemoglobin *and* that normal characteristics could be restored to sickle cells by incorporation of normal hemoglobin, suggesting that interaction of sickle hemoglobin with the cell membrane may be an important determinant of the augmented extensional rigidity of oxygenated sickle cells and plastic-like behavior of the membrane.

METHODS

Cell preparation. Blood was drawn from five patients with homozygous sickle cell disease and two normal volunteer donors into acid citrate dextrose. To isolate cell populations whose densities (and therefore intracellular hemoglobin concentrations) were narrowly defined, erythrocytes were separated by using discontinuous Stractan density gradients (St. Regis Paper Co, Tacoma, WA).[9,10] The density of the Stractan layers ranged from 1.088 to 1.139 g/mL in 0.009-g/mL increments. Erythrocytes trapped between the interfaces of successive layers were removed with a Pasteur pipette, with care to avoid contamination with erythrocytes from the less dense regions of the gradient. The isolated erythrocytes were washed three times with phosphate-buffered saline (PBS: 10 mmoL/L phosphate,

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pH 7.4, 290 mosm/kg) and resuspended in PBS at a hematocrit of 5%. Cell hemoglobin concentration of isolated erythrocyte populations was determined from spectrophotometric measurements of hemoglobin as the cyanomethemoglobin complex and from spun hematocrit.

Hemoglobin solutions for use in reconstitution experiments were prepared by lysing at 0°C 1 vol of well-oxygenated normal or sickle cells washed in pH 6.0 phosphate buffer with 9 vol of deionized water. The pH of the lysed cell suspension was adjusted to be between 6.2 and 6.3 to aggregate cell membranes, and the suspension was centrifuged at 18,000 rpm for five minutes in a Sorvall RC-2B centrifuge. The supernatant hemoglobin solution was carefully removed and concentrated using a sterile Amicon ultrafiltration cell with a DPO2 membrane. The concentrated hemoglobin solutions were freshly made for each of the reconstitution experiments.

Cytoplasmic reconstitution. To assess the relative contribution of altered membrane organization to rheologic properties of sickle erythrocytes, we prepared hybrid erythrocytes consisting of normal erythrocyte membranes reconstituted with sickle hemoglobin and sickle erythrocyte membranes reconstituted with normal hemoglobin using the exchange hemolysis technique described by Clark and Shohet.[11]

In brief, density-fractionated normal or sickle erythrocytes were washed once with PBS and once with pH 6.0, 290 mosm sodium phosphate solution and placed on ice. One milliliter of washed and packed erythrocytes was lysed with 39 mL of ice-cold lysing buffer (10 mmoL/L sodium phosphate, pH 6.0 with 1 mmoL/L EDTA and 0.3 gm/dL dialyzed bovine serum albumin [BSA]). After two minutes of incubation on ice, the membranes were spun down and the supernatant hemoglobin solution removed. Three milliliters of cold-concentrated–donor-hemoglobin solution, previously prepared, was added to the membranes and incubated on ice for five minutes. Small volumes of concentrated adenosine triphosphate (ATP) and $MgCl_2$ solutions were added to the membrane preparations to obtain final concentrations of 1 mmoL/L Mg^{++} and 250 μm ATP. After a sufficient volume of concentrated KCl solution (6900 mosm) was added to restore the tonicity to 290 mosm, the membrane suspension was incubated at 37°C for one hour to promote membrane resealing. The reconstituted hybrid erythrocytes were spun on Stractan density gradients to obtain cells with hemoglobin concentration greater than 28 g/dL.

To verify that the hemoglobin reconstitution procedure did result in loading individual cells with the requisite hemoglobin concentration, a simple optical density method was developed for use with the video microscope system. Absorption of light (at the 436-nm line of a mercury source) was quantitated directly by measurement of the video voltage levels within the cell image, V_c, at the thick toroidal region or rim of the cell compared differentially with the voltage level, V_i, in the clear field outside the cell (both voltages adjusted for dark field levels).* The fractional absorption, $A = (V_c - V_i)/V_i$, was then normalized by the cell thickness to provide a specific absorption, A/d. The cell thickness was measured by manipulation of the cell into an edge-on orientation for observation. The specific absorption was measured on ten to 20 cells obtained from discrete Stractan density fractions both for native red cells and red cells reloaded with various hemoglobin concentrations. The results are cumulated in Fig 1, which represents a "calibration curve" for conversion of optical density to cell hemoglobin concentration with our videomicroscope system. Statistical variations represent uncertainty in cell thickness measurements (because of diffraction limitations) convolved with density variations among cells trapped within a discrete Stractan density level. Even though this video intensity method is not very accurate, it provides a simple approach for simultaneous evaluation of individual cell hemoglobin concentration along with the mechanical tests. Erythrocyte morphology for this range of optical density levels and hemoglobin concentrations is illustrated by the videomicrographs of cells shown in Fig 2. Discoid sickle cells following reconstitution with normal hemoglobin had discoid morphology. Moreover hybrid erythrocytes produced by reconstitution of irreversibly sickled cells with normal hemoglobin continued to exhibit irreversibly sickled shape.

Analytic and experimental approaches to measurement of rheologic properties. Cells deform by extension and folding limited by fixed surface area and volume. As such the static rigidity properties of erythrocytes are quantitated by membrane extension and bending moduli, μ and B; the dynamic rigidities are proportional to the static rigidities multiplied by the time constants measured for recovery from extension (τ_e) and folding (τ_f) deformations divided by the deformation time (ie, the capillary entrance time).[8] Hence because of the rapid entry into small capillaries, dynamic resistance is most likely the dominant impedance to cell transport through the microcirculation. In addition to these viscoelastic properties of the cell, recoverability of the cell shape after deformation characterizes the resilience of the cell; plastic or persistent shape changes following deformation indicate an altered structural arrangement in the membrane that could lead to aberrant cell morphologies such as the irreversibly sickle cells (ISC).

In this study we have focused on the extensional properties of the membrane to evaluate possible "thickening" of the membrane by membrane-associated hemoglobin and resultant changes in membrane properties. For cells with normal densities, the static extensional rigidity, μ, is essentially an elastic modulus that characterizes reversible membrane deformations, ie, the forces that produce extension and recovery follow the same curves with little or no hysteresis.[8,12] For dense cells, on the other hand (especially sickle cells), the rigidity μ is measured as the stiffness coefficient for "loading" or extension of the membrane because there is significant

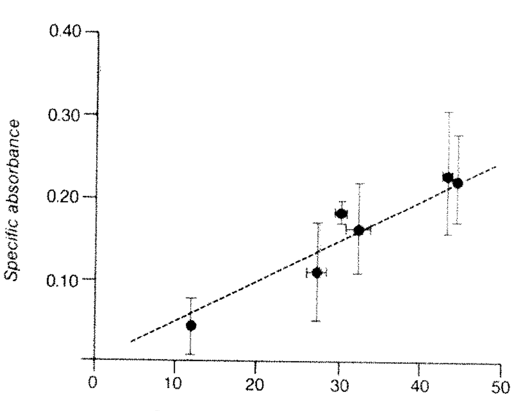

Fig 1. **Plot of specific absorbance v cell hemoglobin concentration in the videomicroscope system. Measurement of specific absorbance of an individual cell enables an estimation of its hemoglobin concentration.**

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*It is important to note that common video cameras have sophisticated automatic amplification circuits. In general, the system adjusts the gain based on the darkest and brightest portions of the image. Hence to employ these cameras as we have, it is necessary to have a portion of the image field dark (eg, provided by a circular aperture just inside video image "box") and to have a constant illumination source (eg, mercury arc lamp). If the illumination is changed or the dark border is absent, then the video signal levels will be internally adjusted.

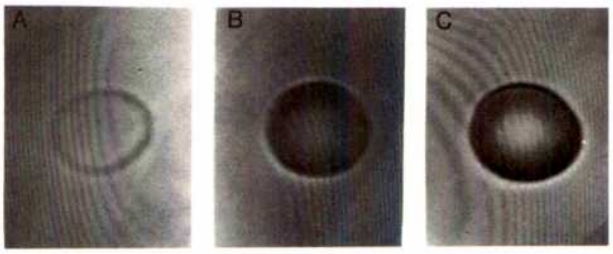

Fig 2. Videomicrographs of reconstituted cells with varying hemoglobin concentrations. The hemoglobin concentration of the cells shown is as follows: (A) 8 g/dL, (B) 30 g/dL, and (C) 34 g/dL.

hysteresis observed as the cell is allowed to recover.[8] After removal of the forces for extension, there is often persistent or residual deformation. These inelastic effects are characteristic of a gel-like material (ie, with a plastic-like yield stress). In the dynamic recovery after cells are released from extensional deformations, the shape recovery process is driven by membrane elasticity but is limited by viscous dissipation in the membrane and in the cytoplasmic and extracellular media. Since the cytoplasmic viscosity is nearly an order of magnitude larger than that of the extracellular medium, the extensional recovery time is approximately given by[13]:

$$\tau_e \sim (\eta_m + \eta_{Hb} \times d/2)/\mu$$

where η_m is the membrane surface viscosity (dyn-s/cm); η_{Hb} is the cytoplasmic viscosity (dyn-s/cm^2); d is the characteristic thickness of the cell. For hemoglobin concentrations from 33 to 40 g/dL, the viscosity ranges from 0.06 to 0.3 dyn-sec/cm^2.[14] Based on a cell thickness dimension of about 2×10^{-4} cm and the membrane elastic modulus measured for normal cells of 6×10^{-3} dyn/cm, we would expect that cells should recover their initial shapes after extension in about 10^{-3} to 10^{-2} seconds for this range of hemoglobin concentrations if there were no membrane dissipation. However, the observed recovery times are 10^{-1} seconds or greater, which indicates that membrane viscous dissipation is dominant and that cytoplasmic viscosity can be neglected. During this study we have tested this conclusion with the use of a cytoplasmic reconstitution method (and by attempting to alter the extent of hemoglobin association with the membrane at constant bulk cytoplasmic hemoglobin concentration). Here it is emphasized that "membrane" includes all intrinsic molecular structures (eg, the amphiphilic molecular double layer or plasmalemma) plus extrinsic or associated structures (eg, the spectrin network or membrane skeleton *and* associated or trapped hemoglobin).

Details of the micromechanical system and tests used to measure erythrocyte mechanical properties have been previously described.[8] Briefly, the membrane extensional rigidity was derived from observation of the cell length aspirated into a small micropipette in proportion to the increase in suction pressure P. Analysis of this experiment has shown that μ is proportional to the derivative of the pressure with respect to length:

$$\mu \propto R_p^2 \times dP/dL$$

where R_p is the pipette inner radius.

The dynamic time constant for recovery τ_e following extensional deformations was derived from tests where erythrocytes were extended end to end by diametrically opposed pipettes. The cells were quickly released and the ratio of length to width was measured as a function of time. The time constant for recovery was derived from correlation of the analytic model for viscoelastic behavior of the red cell membrane with the observed length-to-width recovery.[8,13]

Table 1. Static Extensional Rigidity of Intact Normal and Sickle Erythrocytes as a Function of Cell Hemoglobin Concentration

Erythrocytes	Shear Modulus ($\times 10^{-3}$ dyn/cm)
Normal	
32 g/dL	7.8 ± 0.85
42 g/dL	8.6 ± 1.5
Sickle	
32 g/dL	9.2 ± 2.6
43.3 g/dL	16.5 ± 2.7
44.4 g/dL	21.0 ± 7.8

Values are mean ± SD.

Rheologic properties of 20 intact cells from each density fraction and at least 20 variously reconstituted cells were measured.

RESULTS

Membrane extensional rigidity of native and hybrid erythrocytes. The measured values for static extensional rigidity (μ) of density-fractionated intact normal and sickle erythrocytes are shown in Table 1 for narrowly defined ranges of cell hemoglobin concentration. The shear modulus of normal erythrocytes was independent of cell hemoglobin concentration, while that of oxygenated sickle erythrocytes increased with increasing cell hemoglobin concentration. The shear modulus of sickle erythrocytes with hemoglobin concentration of 44.4 g/dL was 2.5-fold higher than that of either normal erythrocytes or sickle erythrocytes with a hemoglobin concentration of 32 g/dL.

The shear modulus values for variously reconstituted hybrid erythrocytes is shown in Table 2. The elastic shear modulus of normal membranes reconstituted with normal hemoglobin was the same as that of intact normal erythrocytes. In contrast, normal erythrocytes reconstituted with sickle hemoglobin exhibited higher values of extensional rigidity compared to normal membranes reconstituted with normal hemoglobin ($13.1 \; v \; 8.6 \times 10^3$ dyn/cm). The magnitude of the shear modulus value appeared to depend on the hemoglobin concentration of the reconstituted cells. The higher the hemoglobin concentration (as judged by video intensity measurements), the larger the shear modulus of normal erythrocytes reconstituted with sickle hemoglobin. The shear modulus of sickle membranes derived from either low- or high-density sickle cells and reconstituted with normal hemoglobin was very similar to that of normal erythrocytes. The reconstitution of membranes from sickle erythrocytes with a hemoglobin concentration of 44.4 g/dL with normal hemoglobin resulted in the decrease of shear

Table 2. Static Extensional Rigidity of Reconstituted Normal and Sickle Erythrocytes

	Shear Modulus ($\times 10^{-3}$ dyn/cm)
Normal membranes + normal hemoglobin	8.7 ± 1.3
Normal membranes + sickle hemoglobin	13.1 ± 2.9
Sickle membranes from high-density cells (44.4 g/dL) + normal hemoglobin	10.1 ± 2.3

Values are mean ± SD.

modulus value from 21.0 to 10.1 × 10^{-3} dyn/cm. The reconstitution procedure used during these studies was considered successful, since cell morphologies appeared unchanged; also, both optical density and edge-on observation of cells indicated a large hemoglobin uptake; and normal cells reloaded with sickle hemoglobin sickled upon deoxygenation, while sickle cells reloaded with normal hemoglobin did not.

Reversibility of membrane deformation. During the micromechanical measurement of membrane extensional rigidity, we also observed that the curves for aspirated length *v* suction pressure (which are used to derive the value for shear modulus) exhibited different patterns for variously reconstituted cells (Figs 3 and 4). For normal membranes reconstituted with normal hemoglobin, the relationship between aspirated length *v* suction pressure was the same during increasing aspiration pressure (loading phase) and during decreasing aspiration pressure (unloading phase, Fig 3 A). The identical relationship observed between the two variables during both phases of loading and unloading indicates the true elastic behavior of this membrane. In contrast, for normal membranes reconstituted with sickle hemoglobin, the curve of aspirated length *v* suction pressure was markedly different during loading and unloading phases (Fig 3 B). The detection of hysteresis associated with the loading and unloading phases indicates that the normal membrane reconstituted with sickle hemoglobin underwent plastic-like behavior. Similar material behavior was previously seen by us for all sickle erythrocytes irrespective of cell hemoglobin concentration.[8] When reconstituted sickle cells were examined, we found that sickle membranes from the low-density fraction reconstituted with normal hemoglobin exhibited elastic behavior (Fig 4 A), while sickle membranes from the high-density fraction reconstituted with normal hemoglobin exhibited plastic-like behavior (Fig 4 B).

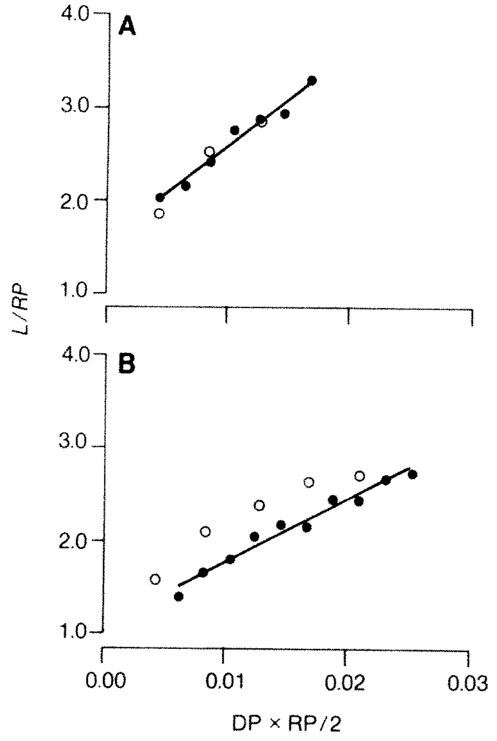

Fig 3. Plot of membrane tension DP × RP/2 (dynes per centimeter, abscissa) *v* the aspiration length normalized by the pipette radius (L/RP) in the micropipette suction experiment. DP is the aspiration pressure; L, the aspirated cell length; and RP, the inner radius of the micropipette. The dark circles are the loading phase of the experiment; the slope of the straight-line fit shown here gives the modulus of extensional rigidity for the cell membrane. The open circles are the unloading phase of the aspiration experiment; the difference between the loading and unloading phases represents the lethargic recovery of the cell membrane after extension for periods of time from two to three minutes. The data shown here are for (A) normal membranes reconstituted with normal hemoglobin; and (B) normal membranes reconstituted with sickle hemoglobin.

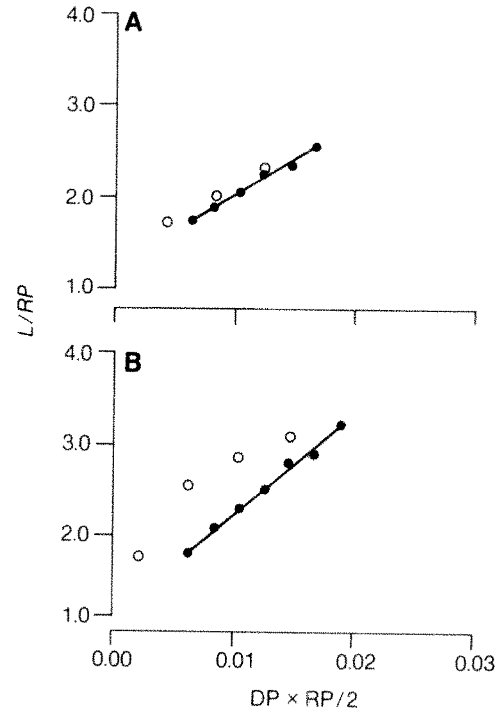

Fig 4. Plot of membrane tension DP × RP/2 (dynes per centimeter, abscissa) *v* the aspiration length normalized by the pipette radius (L/RP) in the micropipette suction experiment. DP is the aspiration pressure; L, the aspirated cell length; and RP, the inner radius of the micropipette. The dark circles are the loading phase of the experiment; the slope of the straight-line fit shown here gives the modulus of extensional rigidity for the cell membrane. The open circles are the unloading phase of the aspiration experiment; the difference between the loading and unloading phases represents the lethargic recovery of the cell membrane after extension for periods of time from two to three minutes. The data shown here are for (A) sickle membranes from the low-density fraction reconstituted with normal hemoglobin; and (B) sickle membranes from the high-density fraction reconstituted with normal hemoglobin.

The plastic-like behavior of the membrane observed during these studies was also reflected by residual, persistent bumps on the membrane surface following the release of the cell from the pipette (Fig 5). To further assess the roles of sickle hemoglobin interaction with the membrane and of alterations in membrane skeletal organization in inducing

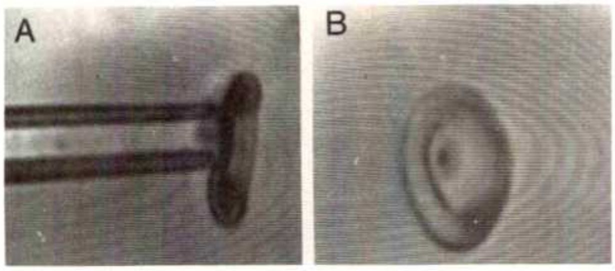

Fig 5. Persistent or residual bumps following mechanical extension. (A) Videomicrograph of pipette aspiration of a normal cell reconstituted with sickle hemoglobin. (B) Videomicrograph of the same cell following its release from the micropipette. The residual, persistent bump on the membrane surface following the release of the cell from the pipette can be clearly seen.

this plastic-like behavior, we determined the fraction of variously reconstituted cells exhibiting persistent bumps following extensional deformation (Table 3). A vast majority (97%) of normal membranes reconstituted with sickle hemoglobin exhibited persistent or residual bumps following extensional deformation, whereas none of the normal membranes reconstituted with normal hemoglobin exhibited such material behavior. Sickle membranes from low-density sickle erythrocytes reconstituted with normal hemoglobin also did not exhibit persistent deformation (3%). Interestingly we found that sickle membranes derived from hyperdense sickle erythrocytes and reconstituted with normal hemoglobin exhibited persistent bumps following extensional deformation (85%).

Dynamic response to extension of native and hybrid erythrocytes. The dynamic response of intact and reconstituted cells to extensional deformation was determined by quantitating the time constant for recovery of the unstressed shape following deformation. To accomplish this, an individual cell was extended end to end by diametrically opposed pipettes such that little buckling or curvature change of the membrane surface took place; then the cell was quickly released and the length-to-width recovery time was analyzed.

Table 3. Residual or Persistent Bumps on Reconstituted Cells Following Extensional Deformation

	No. of Cells Studied	Cells With Residual Bumps	Cells With Residual Bumps (%)
Normal membranes + normal hemoglobin	30	0	0
Normal membranes + sickle hemoglobin	30	29	97
Sickle membranes from low-density cells (32 g/dL) + normal hemoglobin	30	1	3
Sickle membranes from high-density cells (44.4 g/dL) + normal hemoglobin	20	17	85

Table 4. Time Constants for Recovery from Extensional Deformation for Intact and Reconstituted Erythrocytes

	Recovery Time (s)
Sickle erythrocytes	
32 g/dL	0.12 ± 0.03
42 g/dL	0.73 ± 0.19
43.3 g/dL	1.20 ± 0.42
Normal membranes + normal hemoglobin	0.09 ± 0.04
Normal membranes + sickle hemoglobin	0.08 ± 0.02
Sickle membranes from high-density cells (43.3 g/dL) + normal hemoglobin	0.09 ± 0.04

Values are mean ± SD.

The time constant for extensional recovery was derived from the best fit of the viscoelastic model of the observed data.[13] Time constants for recovery from extensional deformation for intact and reconstituted cells are shown in Table 4. The time constant for extensional recovery was strongly dependent on hemoglobin concentration for both normal and sickle erythrocytes, a finding consistent with our previous data.[8] As the cell hemoglobin concentration increased from 32 g/dL to 43.3 g/dL, the recovery times of oxygenated sickle cells increased from 0.12 to 1.2 seconds. The time constant for extensional recovery for variously reconstituted cells was very similar and was slightly less than that measured for low-density normal and sickle cells (0.09 second *v* 0.12 second for intact cells). Normal membranes reconstituted with either normal or sickle hemoglobin and sickle membranes reconstituted with normal hemoglobin all exhibited a time constant for extensional recovery of about 0.09 second. Moreover the time constant for extensional recovery was unaltered when cells were reconstituted with either normal or sickle hemoglobin in the concentration range of 8 to 31 g/dL, although cytoplasmic viscosity is changed by sixfold over this range of hemoglobin concentration.

DISCUSSION

Measurement of rheologic properties of reconstituted normal and sickle erythrocytes has enabled us to identify an important role for sickle hemoglobin interaction with the cell membrane in determining the extensional rigidity and plastic-like behavior of the sickle erythrocyte membrane. Our finding that the shear modulus of normal cells is increased following reconstitution with sickle hemoglobin and that the augmented shear modulus of sickle cells is decreased to normal values following reconstitution with normal hemoglobin implies that sickle hemoglobin interaction with the membrane is a major determinant of increased shear modulus of oxygenated sickle cells. Furthermore the finding that low-density sickle cells no longer exhibit plastic-like behavior following reconstitution with normal hemoglobin and that normal membranes that previously exhibited elastic behavior can be made to undergo plastic deformation following reconstitution with sickle hemoglobin suggests that sickle hemoglobin interaction with the membrane may also be responsible for the plastic-like behavior of intact cells. However, our inability to eliminate the plastic-like behavior of dense sickle cells following reconstitution with normal hemoglobin suggests that either continued association of sickle hemoglobin with the membrane or irreversible alterations in skeletal protein associations may also contribute to the plastic deformation of the subpopulation of sickle cells.

Increased association of sickle hemoglobin with the erythrocyte membrane has previously been documented by a number of investigators.[15-21] Using the spin-label electron paramagnetic resonance technique, Fung et al[18] and Fung and Lilley[21] showed enhanced binding of sickle hemoglobin to membrane at physiologic pH when compared to normal hemoglobin. Eisinger et al,[20] using resonance energy transfer measurements on intact, oxygenated sickle erythrocytes, showed that concentration of sickle hemoglobin proximal to the membrane (h_b) was significantly higher than the bulk hemoglobin concentration in the cytoplasm (h_c) and that the ratio of these two concentrations (h_b/h_c) increased from 1.0 to 1.5 as cell hemoglobin concentration increased from 34 g/dL to 45 g/dL. This increase in sickle hemoglobin concentration near the membrane was not seen in ghosts. In contrast, in normal erythrocytes, hemoglobin concentration proximal to the membrane was less than the bulk hemoglobin concentration ($h_b/h_c = 0.7$); and the ratio of h_b to h_c did not change as the cell hemoglobin concentration increased from 34 to 42 g/dL. These findings of a concentration-dependent increase in the association of sickle hemoglobin but not of normal hemoglobin with the membrane, as well as the reversible nature of the hemoglobin interaction with the membrane, are consistent with our rheologic data. The hemoglobin binding data lend strong support to our suggestion that the augmented extensional rigidity of sickle cells and their increased tendency to undergo plastic-like behavior may be mediated by the increased interaction of sickle hemoglobin with the membrane.

The finding that dense dehydrated sickle cells continue to exhibit plastic-like behavior following reconstitution with normal hemoglobin implies that either strong association of sickle hemoglobin with these membranes or alterations in normal membrane skeletal protein organization may be responsible for this type of material behavior. Biochemical evidence to support both these types of membrane changes has previously been identified in dense dehydrated sickle cells.[22-25] With the present data it is difficult to determine the relative contributions of these two membrane alterations to the observed material behavior of dense sickle cells. It should be noted, however, that following reconstitution with normal hemoglobin the extensional rigidity of this subpopulation of sickle cells showed marked decrease, implying that reversible association of sickle hemoglobin with the membrane is still the dominant cause of the increased static rigidity of dense sickle cells.

The dynamic rigidity of intact normal and sickle cells, quantitated by measuring time constants for extensional recovery, was found to be strongly dependent on cell hemoglobin concentration, data consistent with our prior observations.[8] The finding that the time constant for extensional recovery was unaltered when cells were reconstituted with either normal or sickle hemoglobin in the concentration range of 8 to 31 g/dL while the cytoplasmic viscosity is changed by sixfold over this range of hemoglobin concentration lends support to earlier theoretical calculations that membrane viscosity and not cytoplasmic viscosity is the key determinant of energy dissipation during recovery from extensional deformation.[4,13,26] The marginal decrease in extensional recovery times for reconstituted cells compared to intact cells (0.09 second *v* 0.12 second) suggests loss of some membrane-bound material during the lysing and reloading procedure. Similar decreases in recovery times for ghost membranes have previously been seen by other investigators.[27]

From our current studies it is clear that rheologic properties of oxygenated sickle cells are strongly influenced by hemoglobin interaction with the cell membrane. The augmented extensional rigidity of sickle cells and their increased tendency to undergo plastic deformation appears to be the result of increased concentration of sickle hemoglobin adjacent to the membrane. The data we have obtained also provide some new insights into the biophysical consequences of the previously described interaction between sickle hemoglobin and cell membrane.

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c-fos Oncogene Expression in Human Hematopoietic Malignancies Is Restricted to Acute Leukemias With Monocytic Phenotype and to Subsets of B Cell Leukemias

By Antonio Pinto, Giulia Colletta, Luigi Del Vecchio, Rita Rosati, Vincenza Attadia,
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To evaluate relationships between *c-fos* proto-oncogene expression and specific lineages of hematopoietic differentiation we analyzed the constitutive and TPA-induced expression of the *c-fos* gene in a wide variety of fresh human leukemic cells.

High constitutive *c-fos* expression was detected in acute leukemias with monocytic phenotype (FAB M4/M5) and in subsets of B lymphoid leukemias, some of which coexpressed B lymphocytic and monocytic markers. Conversely, low basal levels of *c-fos* transcripts were found in pure acute granulocytic leukemias (FAB M1/M2/M3), in erythroleukemias (FAB M6), in the great majority of B, and in all T lymphoid leukemias. TPA-induced *c-fos* expression seems to correlate with monocytoid differentiation only when sustained levels of transcripts (ie, detectable for at least 24 hours) were detected. Sustained *c-fos* expression was in fact observed only in those myeloid or lymphoid cells that acquired a stable monocyte-like phenotype in response to the phorbol ester. These results indicate that high constitutive *c-fos* expression may identify myelomonocytic-oriented forms of leukemia, specific subsets of B lymphoid malignancies, and at least some cells terminally differentiated in vitro to a monocyte-like phenotype. *c-fos* oncogene expression can therefore be regarded as an additional marker for the subclassification of human leukemias.

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CHANGES in oncogene expression have been described in hematopoietic cells as linked to cell growth or differentiation.[1-3] In vitro induction of differentiation of murine and human leukemic cells by agents such as dimethylsulfoxide (DMSO),[4-6] retinoic acid (RA),[4] tetradecanoil phorbol acetate (TPA),[6,7] 1,25-$(OH)_2$ D_3,[8] hexamethylenebisacetamide (HMBA),[9] or physiologic regulators like colony-stimulating factor (CSF),[10,11] is in fact associated with a decreased expression of the previously overexpressed oncogenes *c-myc* and *c-myb*. These changes have been related to the observed inhibition of cell proliferation rather than to the induction of differentiation toward a specific lineage. Only recently a number of reports have appeared that link the expression of cellular oncogenes to a specific pathway of hematopoietic differentiation. Increasing amounts of two cellular oncogene transcripts, *c-fos* and *c-fms*, accumulate in hematopoietic cells as the monocytic maturation proceeds.[12-14] Induction of differentiation of murine myelomonocytic cells WEHI-3 into macrophages by CSF and actinomycin D is accompanied by a remarkable increase in *c-fos* transcripts, maximal three days after induction.[10] Furthermore a rapid induction of the *c-fos* oncogene has been reported during TPA-induced monocytic differentiation in human cell lines HL-60[12,14] and U-937.[15] In this case the kinetics of induction was quite different, since *c-fos* transcripts reach their maximal levels 30′ to 40′ following TPA exposure and decrease rapidly thereafter to levels significantly superior to those of uninduced cells. Accordingly, high levels of *c-fos* RNA transcripts have been detected in primary cultures of mouse peritoneal macrophages.[12] On the other hand, no increase in *c-fos* transcripts was observed when HL-60 cells were induced to differentiate into granulocytes by DMSO.[15] These observations suggest that *c-fos* expression is associated with differentiation along the monocyte/macrophage pathway and that the *fos* oncogene may play a role in different events occurring during monocytic maturation.[15,16]

Most of these studies have been conducted on established leukemic cell lines, adapted to grow in vitro for a long time, whose behavior may not necessarily reflect all events occurring in leukemic cells in vivo. Conversely, the analysis of fresh cell samples from a wide variety of leukemias, a disease regarded as a clonal expansion of cells reflecting the phenotypes of normal hematopoietic cells at various stages of maturation,[17,18] may represent a more useful approach by which to investigate the lineage-specific expression of the fos oncogene and may provide some hints on the pathophysiology of hematologic malignancies. Our results show that constitutive *c-fos* expression in human hematopoietic tumors is restricted to myeloid cells displaying markers of monocytic differentiation and to subsets of B cell malignancies, some of which coexpress lymphocytic and monocytic markers.

MATERIALS AND METHODS

Isolation of leukemic cells. Fresh leukemic cells were obtained from the peripheral blood or bone marrow of patients diagnosed as having acute nonlymphoid leukemia (ANNL), acute lymphoid

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leukemia (ALL), chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), and hairy cell leukemia (HCL). All samples were collected before chemotherapy and after informed consent. Diagnoses were based on cell morphology plus standard cytochemical stainings, and acute leukemias were classified according to the French-American-British (FAB) criteria[19] in acute myeloid/granulocytic leukemia (AML; FAB M1, M2, M3), acute myelomonocytic leukemia (AMML; FAB M4), acute monocytic leukemia (AMoL; FAB M5), and acute erythroleukemia (AEL; FAB M6). Only patients who had at least 80% of leukemic cells in peripheral blood or bone marrow were chosen as donors.

Mononuclear cells were purified on a Ficoll-Hypaque (FH) gradient and subjected to two cycles of adherence on a plastic culture dish for one hour at 37°C. Such procedure yielded a population of leukemic cells more than 90% pure and virtually free of contaminating normal monocytes as revealed by May-Grünwald-Giemsa (MGG) and cytochemical stainings of cytospin preparations. Leukemic cells were processed for RNA extraction soon after Ficoll purification to evaluate constitutive *c-fos* expression or were cultured in the presence of TPA as described below.

Culture conditions and in vitro differentiation. Fresh leukemic cells were seeded at a concentration of 1×10^6/mL in tissue culture dishes (Falcon) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories Inc, Irvine Ayrshine, UK) and 4 mmol/L glutamine. TPA (Sigma Chemical Co, St. Louis) dissolved in DMSO was added to a final concentration of 20 ng/mL. Control cultures were grown in 0.002% DMSO. After 30' or 24 hours of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, control and TPA-treated cells were collected, washed twice in cold RPMI, and processed for RNA extraction as described below. Control and TPA-treated myeloid cells were stained for sodium fluoride (NaF) inhibitable naphthyl-acetate-esterase (α-NAE) and napthol-ASD-chloroacetate esterase (N-ASD-CAE) as previously described.[20] The percentage of adherent cells in myeloid leukemic cells following TPA exposure was evaluated as previously reported.[21] U-937 cells were maintained in RPMI 1640 complete medium and subcultured twice weekly.

Immunophenotypic characterization of leukemic cells. Leukemic cells were characterized by a panel of monoclonal antibodies (MoAbs) either immediately after Ficoll purification or after culture in the presence or absence of TPA. MoAbs recognizing antigenic specificities of different blood cell lineages and grouped into "clusters of differentiation" (CD) according to criteria of the III International Conference and Workshop on Leukocyte Antigens (Oxford, 1986) were either a gift of Dr G. Rovera from Wistar Institute, Philadelphia (S5.25, S5.7)[22] or were obtained through commercial sources: OKM1, OKB2, OKT11, OKT6, OKT8, OKT3, OKT4 (Ortho Pharmaceuticals Corp, Raritan, NJ); anti-HLA-DR, Leu-10 (HLA-DQ), Leu-1, anti-IL-2R (interleukin 2 receptor), anti-TrR (transferrin receptor) (Becton Dickinson Co, Sunnyvale, CA); B4, MO2, MY7, MY9 (Coulter Immunology, Hialeah, FL); BA-2 (p24 Ag), 33.1 (HLA-DQ), BA-3 (CALLA) (Hybritech Inc, San Diego); fluorescein-conjugated (FITC)-conjugated goat $(FAB')_2$ antihuman Ig, IgA, IgM, IgD, IgG, k (Kallestad, Austin, TX). In all cases 0.5×10^6 leukemic cells in a volume of 100 μL were incubated at 4°C for 30' with different MoABs at the appropriate dilution. Cells were then washed three times with phosphate-buffered saline (PBS) and incubated at 4°C for an additional 30' with FITC-conjugated goat antimouse Ig (Ortho). After three more washings in PBS cells were analyzed by means of a flow cytometer (Ortho, Spectrum, IL). For cytoplasmic Ig (CyIg) analysis, cytospin preparation were fixed in methanol/acetone (3:1) for 5' at -20°C prior to FITC-conjugated goat antihuman IgM application. Cells were then observed under an epifluorescence UV microscope (Zeiss).

RNA extraction and hybridization. Total cellular RNA was extracted from leukemic cells either soon after Ficoll purification or after 30' and 24 hours of culture in the presence of 20 ng/mL TPA by the guanidium hydrochloride method.[23] Total RNA (5 μg/lane) was fractionated on 1% agarose gel containing 2.2 mol/L formaldehyde in MOPS buffer and transferred onto nitrocellulose filter.

The RNA bound to nitrocellulose was prehybridized for four hours at 42°C (in 1% glycine, 40% formamide, 5X SSC, 50 mmol/L NaH_2PO_4 pH 6.5, 250 μg/mL sonicated salmon sperm DNA, 1X Denhardt's solution and hybridized for 12 to 16 hours at 42°C (in 50% formamide, 2X SSC, 40 mmol/L NaH_2PO_4 pH 6.5, 200 μg/mL salmon sperm DNA, 10% dextran sulphate, 10 mmol/L EDTA, 0.1% SDS) with 3×10^6 cpm of ^{32}P nick-translated purified DNA fragments (2×10^8 cpm/μg of DNA). The oncogene-specific probes used were *v-fos* (pBR322 1.0 Kb PstI insert)[24] and v-ras (clone HiHi 3 pBR322 1.0 Kb Hinc II fragment).[25] The size of the *c-fos* transcripts was determined relatively to 18S and 28S rRNA markers, which were assumed to correspond to 1.8 and 4.5 Kb, respectively. For dot-blot hybridization studies different amounts of total cellular RNA were denatured and diluted in 10X SSC and spotted onto nitrocellulose by means of a dot-manifold apparatus (Schleicher & Schuell). Filters were than baked and hybridized to 6×10^6 cpm of oncogene-specific ^{32}P nick-translated probes as described for Northern blots. Filters were washed two times in 2X SSC at room temperature for 20 minutes, two times in 0.5X SSC, 0.1% sodium dedecyl sulfate (SDS) at 50°C for 30 minutes, and finally four times in 2X SSC at room temperature for ten minutes. Levels of *c-fos* RNA were expressed as arbitrary units (au) relative to the levels of *c-fos* RNA present in uninduced U-937 cell line set arbitrarily to 1.0. The specificity of the hybridization experiments with the *c-fos* probe and the possibility of RNA miscalibration or of a TPA-induced aspecific increased or decreased transcription were assessed by hybridizing the same blots with a different probe specific for the *c-ras*^{ki} oncogene that represented the internal calibration of the system.

RESULTS

Basal expression of the c-fos *oncogene in myeloid cells.* Constitutive expression of *c-fos* oncogene in human myeloid cells was investigated by evaluating the presence of *c-fos*-specific transcripts in leukemic samples from 16 patients with various forms of ANLL. An example of dot-hybridization assay of RNA extracted from myeloid leukemias is shown in Fig 1. Cells obtained from leukemia subtypes FAB M4 and M5 expressed levels of *c-fos* up to tenfold higher than those detectable in other FAB subtypes. Conversely *c-ras*^{ki} expression did not show any significant variation among the different leukemia samples (Fig 1).

In Table 1 relative levels of *c-fos* expression in all the 17 leukemic samples (both peripheral blood and bone marrow were examined in patient No. 2) are reported, along with α-NAE activity and OKM1/CD11b and MO2/CD14 MoAbs binding. The range of *c-fos* expression within the AMML/AMol group (FAB M4, M5) varied from 3.8 to 9.9 au. On the other hand, pure granulocytic leukemias (FAB M1, M2, M3) and the two erythroleukemic samples (FAB M6) studied expressed lower levels of *c-fos* transcripts (0.2 to 1.9 au).

By comparing the levels of *c-fos* expression with the differentiation markers on leukemic cells, it appears that there was a strong correlation between the presence of "true" monocytic markers (α-NAE and MO2/CD14) and high

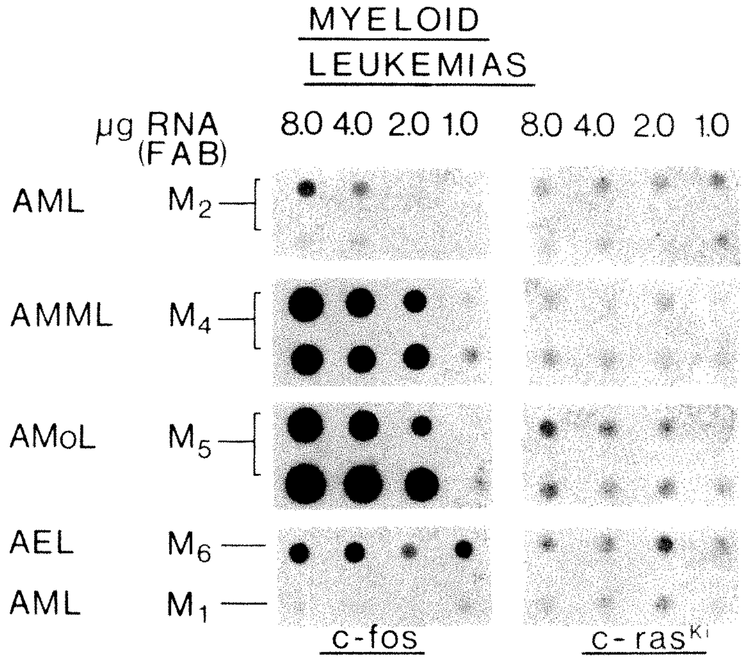

Fig 1. Basal levels of *c-fos* and *c-ras*^{ki} oncogene expression in human myeloid leukemia cells as detected by dot-blot hybridization. RNAs were obtained from the following samples (see Table 1): AML FAB M2 (patient no. 1, patient no. 2/PB), AMML FAB M4 (patient no. 8, patient no. 10), AMoL FAB M5 (patient no. 11, patient no. 14), AEL FAB M6 (patient no. 15), AML FAB M1 (patient no. 3). For details on RNA isolation and hybridization, see Materials and Methods.

Table 1. *c-fos* Expression in Human Myeloid Leukemic Cells

Patient No.	(FAB)	Cell Source	OKM1	MO2	α-NAE	c-fos (au)
1	AML M2	(PB)	28*	2	7†	1.9‡
2	AML M2	(PB)	48	10	10	0.9
2	AML M2	(BM)	33	NT	11	2.0
3	AML M1	(PB)	3	1	8	0.4
4	AML M2	(PB)	30	35	12	0.3
5	AML M3	(BM)	18	1	NT	0.2
6	AML M2	(PB)	22	12	NT	0.7
7	AMML M4	(PB)	36	48	51	6.1
8	AMML M4	(PB)	40	56	38	4.0
9	AMML M4	(BM)	34	NT	50	3.8
10	AMML M4	(PB)	18	50	48	4.6
11	AMoL M5	(PB)	20	57	78	4.1
12	AMoL M5	(BM)	80	69	80	6.2
13	AMoL M5	(PB)	60	58	70	8.9
14	AMoL M5	(PB)	43	61	63	9.9
15	AEL M6	(BM)	15	NT	6	1.8
16	AEL M6	(BM)	28	1	NT	1.2

Abbreviations: α-NAE, α-naphthyl acetate esterase; AML, acute myeloid leukemia (FAB M1, M2, M3); AMML, acute myelomonocytic leukemia (FAB M4); AMoL, acute monocytic leukemia (FAB M5); AEL, acute erythroleukemia (FAB M6); PB, peripheral blood; BM, bone marrow; NT, not tested.

*Percent of positive cells determined by indirect immunofluorescence using an Ortho Spectrum III flow cytometer. Fresh leukemic cells were analyzed soon after Ficoll purification.

†Percent of positive cells. Fresh leukemic cells were analyzed soon after Ficoll purification.

‡Levels of RNA quantitated by dot-blot hybridization followed by densitometric scanning of autoradiographic spots and expressed as arbitrary units (au) relative to the levels of *c-fos* RNA present in uninduced U-937 cell line set arbitrarily to 1.0. Values > 2 au are encased.

levels of *c-fos* expression. On the contrary, no correlation was found between *c-fos* expression and the panmyeloid antigen (CD11b) recognized by the OKM1 antibody. The restriction of constitutive *c-fos* expression to cells belonging to the monocytic lineage indicates that basal levels of *c-fos* transcription might be directly related to the presence on leukemic cells of monocyte-restricted markers such as sodium fluoride inhibitable α-NAE and the binding of the MO2/CD14 antibody.

Basal expression of c-fos *oncogene in lymphoid cells.* *c-fos* expression was evaluated as described for myeloid cells; an example of dot-blot hybridization of RNA from lymphoid malignancies with a *v-fos* and a *v-ras*^{ki} probe is shown in Fig 2. *c-ras*^{ki} RNA levels did not show any appreciable variation among the different leukemias examined. On the contrary, some of the samples revealed levels of *c-fos* expression up to sixfold higher than those detectable in other leukemic cells of the same subtype. In Table 2 relative levels of *c-fos* expression in all the 17 samples of lymphoid leukemias studied are shown, along with the phenotypic characterization performed with MoAbs against lymphoid and myeloid antigens. Low levels of *c-fos* expression (≤2 au) were detected in 11 of 17 samples without specific distribution relatively to the lineage or stage of differentation. Higher levels, comparable to those of myelomonocytic leukemias, were observed in six cases, all of B lineage. Among those, B CLL patients No. 26 and 27 displayed, together with lymphoid markers, at least four monocytic antigen (OKM1/MO1/CD11b, MY7/CDw13, and MY4/CD14); the pre-pre B leukemia harboring the t(4;11) translocation, patient No. 20, showed positivity for four monocytic markers (OKM1/MO1/CD11b, MY9/CD36, MY7/CDw13) and α-NAE. Cells from a patient with HCL displaying positivity for the OKM1 and Leu-M5 markers also highly expressed *c-fos* transcripts. Among lymphatic leukemias without concomitant expression of monocytic markers, high level of *c-fos* transcripts

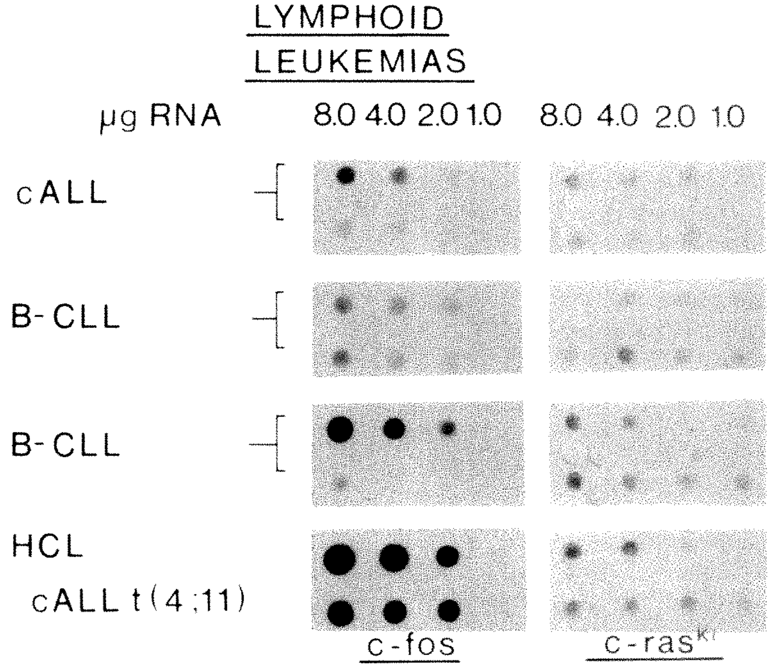

Fig 2. Basal levels of *c-fos* and *c-ras*^{ki} oncogene expression in human lymphoid leukemia cells as detected by dot-blot hybridization. RNAs were obtained from the following samples (see Table 2): cALL (patient no. 18, patient no. 19), B CLL (patient no. 24, patient no. 25), B CLL (patient no. 26, patient no. 23), HCL (patient no. 29), CALL t(4,11) (patient no. 20). For details on RNA isolation and hybridization, see Materials and Methods.

were detected only in the most immature of the cases examined, ie, a B-committed ALL (patient No. 17) and in cells from a PLL (patient No. 28). Both acute and chronic T lymphoid leukemias showed a *c-fos* expression slightly higher than uninduced U-937 cells (<2.0 au).

c-fos *expression during TPA-induced monocytic and lymphoid differentiation.* Cells from 22 patients with different forms of leukemias (Table 3) were exposed in vitro to 20 ng/mL TPA, and total cellular RNA was extracted at 30′ and 24 hours and hybridized to *fos* and *ras*^{ki} probes. Specific

Table 2. *c-fos* Expression in Human Lymphoid Leukemic Cells

Patient No.		Cell Source	FAB	B4	CALLA	CyIg	SmIg	HLA-DR	OKM1	MO1	MY4	MY7	MY9	α-NAE	*c-fos*
17	B committed	(PB)	L1	29*	1	0	4	42	NT	5	1	2	1	NT	$\boxed{3.5}$
18	Pre-Pre B	(PB)	L2	72	64	3	2	73	NT	2	3	5	7	NT	2.0
19	Pre-Pre B	(PB)	L2	55	24	1	5	60	NT	6	NT	2	2	12†	1.0
20	Pre-Pre B‡	(PB)	L2	91	88	2	1	86	39	46	1	41	39	61	$\boxed{6.0}$
21	pre-B	(PB)	L1	85	62	30	1	90	1	NT	NT	6	5	16	1.7
21	pre-B	(BM)	L1	91	50	36	2	94	NT	NT	NT	NT	NT	NT	2.0
22	B	(PB)	L3	42	0	2	83	80	NT	2	2	NT	3	9	0.4
23	B CLL	(PB)	—	71	1	NT	30	72	5	3	3	2	NT	21	0.5
24	B CLL	(PB)	—	69	0	NT	32	83	10	11	1	9	3	16	1.3
25	B CLL	(PB)	—	68	1	NT	41	84	NT	7	10	6	10	25	1.4
26	B CLL	(PB)	—	90	1	NT	33	84	58	55	28	36	3	19	$\boxed{4.5}$
27	B CLL	(PB)	—	81	0	NT	45	86	78	81	37	43	5	39	$\boxed{5.6}$
28	B PLL	(PB)	—	80	0	NT	86	82	15	NT	1	5	NT	11	$\boxed{4.1}$
29	B HCL§	(Sp)	—	64	NT	NT	71	80	90	72	6	9	NT	21	$\boxed{6.5}$
				OKT11	Leu-1	OKT6	OKT3	OKT4	OKT8						
30	T ALL	(PB)	L1	9	89	90	1	8	45						2.0
31	T ALL	(PB)	L1	77	83	1	2	1	2						1.8
32	T CLL	(PB)	—	35	22	2	80	17	55						2.0

Abbreviations: CyIg, cytoplasmic immunoglobulins; SmIg, membrane immunoglobulins; α-NAE, α-naphthyl acetate esterase; ALL, acute lymphoid leukemia; CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; HCL, hairy cell leukemia; PB, peripheral blood; BM, bone marrow; Sp, spleen; NT, not tested.

*Percent of positive cell determined by indirect immunofluorescence using an Ortho Spectrum III flow cytometer. Fresh leukemic cells were analyzed soon after purification.

†Percent of positive cells. Fresh leukemic cell were analyzed soon after purification. Relative levels of *c-fos* RNA quantitated by dot-blot hybridization followed by densitometric scanning of autoradiographic spots and expressed as arbitrary units (au). Values > 2 au are encased.

‡t(4;11) translocation.

§89% Leu-M5 positive cells.

Table 3. c-fos Expression in Human Leukemic Cells During TPA-Induced Differentiation

Patient No.		TPA	
		30′	24 h
1	AML	13.6*	6.5
2	AML	2.8	2.0
3	AML	0.1	0.8
7	AMML	24.3	13.0
8	AMML	4.8	2.8
11	AMoL	0.02	1.0
13	AMoL	6.3	3.2
15	AEL	2.2	1.2
17	B committed ALL	4.4	0.05
19	Pre-Pre B ALL	1.3	0.6
20	Pre-Pre B ALL†	3.2	2.2
21	Pre B ALL	3.1	1.1
23	B CLL	2.1	1.1
24	B CLL	21.0	1.0
25	B CLL	7.9	0.01
26	B CLL	2.5	2.9
27	B CLL	11.2	4.0
28	B PLL	4.8	0.02
29	B HCL	1.1	1.0
30	T ALL	0.03	0.9
31	T ALL	0.2	1.1
32	T CLL	0.005	1.0

Abbreviations: AML, acute myeloid leukemia (FAB M2); AMML, acute myelomonocytic leukemia (FAB M4); AMoL, acute lymphoid leukemia (FAB L1, L2); CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; HCL, hairy cell leukemia.

*Relative levels of *c-fos* RNA in leukemic cells exposed for 30 minutes and 24 hours to 20 ng/mL TPA. RNA levels have been quantitated by densitometric scanning of autoradiographic films and are expressed as folds of increase relatively to the levels of uninduced cells (ie, cells cultured for 30 minutes and 24 hours in TPA-free medium).

†t(4,11) translocation.

RNA levels were analyzed by Northern blotting hybridization and quantitated by densitometric scanning of dot-blot autoradiographic films and expressed as times of increase relative to levels of uninduced cells. Addition of TPA to six of 8 myeloid leukemia cell cultures resulted in an overall rapid increase (up to 24.3-fold) in *c-fos* transcripts, which was maximal after 30′ and which decreased thereafter, reaching at 24 hours levels superior (twofold to 13-fold) to those of the uninduced cells (Table 3). Unexpectedly, a drastic reduction of *c-fos* transcripts following TPA treatment was observed in cells from patients No. 3 (FAB M1) and No. 11 (FAB M5). A representative Northern blot showing the major specific 2.2 Kb *c-fos* band in FAB M2 cells after TPA exposure is given in Fig 3. RNA extracted from myeloid leukemic cells at later times (up to five days) following TPA induction showed levels of *c-fos* threefold to fivefold those of uninduced cells (data not shown). *c-ras*^{ki} levels did not change significantly after TPA induction (data not shown). At three days after TPA addition, up to 70% to 90% of the cells of all myeloid leukemia patients, except patient No. 3, displayed strong adherence to plastic surface. In addition, in all AML (FAB M1, M2) and AMML (FAB M4) samples, the percentage of cells showing NaF inhibitable α-NAE activity increased sharply three days after TPA exposure, whereas N-ASD-CAE activity was reduced in all AML and AMML cells except in the case of patient No. 3. The expression of myelomonocytic differentation antigens was also modulated by TPA. The reactivity with the OKM1/CD11b and S5.25 antibodies, both reactive with late myeloid cells and mature monocytes, was in fact greatly increased in myeloid leukemia samples (FAB M2, M4, M5). The reactivity with the monocyte-restricted antibody MO2/CD14 increased in AMML/AMoL cells or was induced in AML cells following TPA treatment, whereas the expression of the immature myeloid antigen recognized by the S5.7 antibody was generally decreased (data not shown). Interestingly, as seen for the other parameters evaluated, TPA did not affect the antigenic pattern of cells from patients No. 3 and 11, whose immature phenotype remained unmodified.

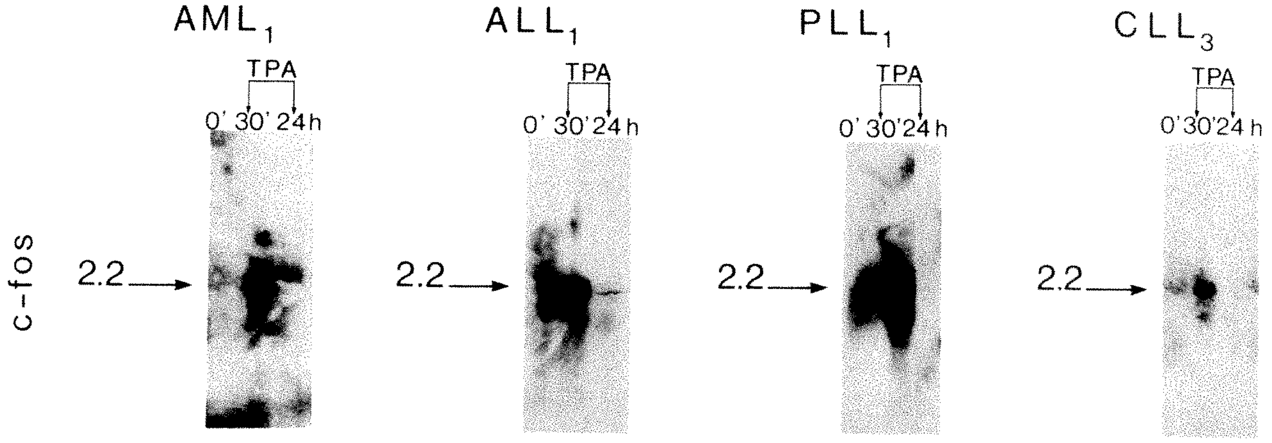

Fig 3. Expression of *c-fos* oncogene in human leukemia cells before and after TPA-induced differentiation as detected by Northern blot hybridization. RNAs were extracted after 30 minutes or 24 hours of exposure to TPA (20 ng/mL). RNAs in the 0′ lanes were obtained from cells grown for 30 minutes in TPA-free culture medium. RNAs were obtained from the following samples: AML 1 (patient no. 1), ALL 1 (patient no. 17), PLL1 (patient no. 28), and CLL3 (patient no. 25). For details on in vitro induction of differentiation, RNA isolation, blotting and hybridization, see Materials and Methods.

Treatment with 20 ng/mL TPA of cell from various forms of lymphoid leukemias resulted in a general induction of *c-fos* expression only in B cell malignancies. As the representative Northern blot in Fig 3 shows, the specific 2.2 Kb *c-fos* band, already present at high basal levels in ALL1, PLL1, and to a lesser extent in B CLL3 (patients No. 17, 28, and 25 respectively), increased after 30′ of TPA treatment and disappeared at 24 hours. Relative levels of *c-fos* expression in lymphoid leukemias quantitated from dot blots and indicated as fold of increase compared to the untreated controls are shown in Table 3. On the whole, TPA induced *c-fos* expression in nine of 11 samples of B lymphoid leukemias, whereas a drastic reduction in levels of *c-fos* was triggered by TPA in all three T cell malignancies. In the HCL cells, *c-fos* levels, already constitutively high, were unaffected by exposure to phorbol ester. The degree of induction in B cell leukemias ranged between 2.1-fold and 21-fold. After 24 hours of TPA treatment *c-fos* transcription returned to levels equal to or lower than those detected prior to induction, with the exception of cells from patients No. 20, 26, and 27.

TPA treatment also resulted in the modification of the surface phenotypes in B and T cell leukemias. Antigens of B lineage differentiation such as HLA-DR, HLA-DQ, and B4 were either increased in intensity or induced. B cell activation antigens, such as IL 2 receptor, 33.1 (DQ-like) and BA2 (p24 Ag), were all induced by TPA, whereas antigens characteristic of immature stages of B cell differentiation, such as OKB2, disappeared following treatment. These changes are suggestive of a TPA-induced differentiation of leukemic cells toward mature elements of the B lineage. The myelomonocytic markers (OKM1/MO1/CD11b, MY7/CDw13, MY4/CD14, Leu-M5/CD11d) detected in the subset of leukemias with high constitutive *c-fos* expression were similarly increased after TPA treatment along with the induction of the monocyte-restricted antigen MO2/CD14 and of markers of B lineage activation/differentiation (IL 2R, HLA-DR, HLA-DQ, BA2, and B4). The two acute T cell leukemias responded to TPA with the disappearance of the immature T lymphocyte antigen (OKT6) and with an increased intensity of the pan-T molecules T11 and T1. In the T CLL, a remarkable increase in both percentage of positivity and intensity of T11 and T1 and decreased T4 expression were induced. In all the T cell leukemias the receptor for IL 2 was strongly induced by TPA. These changes are compatible with differentiation into mature T lymphocytes.

DISCUSSION

Recent studies have suggested that the product of the *c-fos* gene may be involved in the differentiation of cells in the monocytic lineage.[12-16] High levels of *fos* transcripts have been found in primary cultures of normal murine macrophages,[12] and *c-fos* transcription and *c-fos* protein were both induced when human (U-937, HL-60) and murine (WEHI-3) leukemic cell lines were triggered to differentiate into macrophages by agents such as TPA[12,14,15] and CSF.[10] Nevertheless transient activation of *c-fos* transcription was shown to be in itself insufficient to commit U-937 cells to differentiate when stimulated by serum or diacylglycerol (DAG), and differentiation of HL-60 to macrophages by 1,25-$(OH)_2D_3$ was not accompanied by *c-fos* induction.[26] Therefore the role of *c-fos* expression in the differentiation of hematopoietic cells remains unclear. On the other hand, the definition of the lineage-specific expression of the *fos* proto-oncogene may be important in understanding hematopoietic differentiation and may provide new insights into the pathophysiology of leukemias. Consequently *c-fos* expression was studied at basal levels and after treatment with TPA in a wide variety of fresh cell from patients with different hematopoietic malignancies representing different stages and lineages of blood cell differentiation.[17,18] Our results show that a clear connection can be traced between *c-fos* expression and monocytic differentiation. In fact, high basal levels of *c-fos* transcripts were detected in myeloid leukemic cells showing markers of monocytic differentiation (FAB M4, M5), whereas very low amounts of transcripts, if any, were found in granulocytic oriented forms of myeloid leukemias (FAB M1, M2, M3) and in erythroleukemia cells (FAB M6). The expression of the monocytic-restricted antigen MO2/CD14 but not that of the panmyeloid OKM1/CD11b strongly correlated with higher *c-fos* RNA levels. This correlation was also supported by the results obtained after TPA treatment of ANLL cells in which differentiation along the monocytic pathway was accompanied by a remarkable increase in *c-fos* transcripts, resembling the kinetics of induction already described for TPA-treated U-937[15] and HL-60[14] cells. In fact, also in our hands a sustained expression of the *c-fos* gene, detectable for at least 24 hours, was found in those samples inducible to monocytic differentiation by TPA. That monocytic differentiation and *c-fos* expression are correlated was also suggested by the finding that in the two myeloid leukemic samples that did not show any differentiation in response to TPA, no increase in *c-fos* levels was induced by the phorbol ester.

The lineage-specificity of *c-fos* expression was strengthened by the low levels of constitutive expression found in lymphoid malignancies displaying pure B and T lymphocyte markers. Surprisingly, higher basal levels, comparable to those of monocytic-oriented forms of ANLL, were found in a group of lymphatic leukemia cells coexpressing B lymphocytic and myelomonocytic antigenic markers and also in the most undifferentiated form of ALL, a B-committed ALL, and the most immature form of chronic lymphoid leukemia, a PLL.

A rapid and transient *c-fos* expression was induced by TPA in almost all (nine of 11) B lymphoid samples, independently of the basal level of expression, and was accompanied by the induction of markers of B cell activation/differentiation. Yet sustained levels of *c-fos* expression were detected only in those cases within B cell leukemias in which induction of myelomonocytic markers was detected after TPA treatment.

The correlation between sustained *c-fos* expression and monocytic differentiation also seems to be confirmed by different data showing that treatment of both human leukemic cell lines and primary cultures with cytosine arabinoside and 5-aza-2′-deoxycytidine, which exert a molecular mechanism of action different from TPA, results, along with the appearance of a monocytic phenotype, in a progressive increase of steady-state levels of *c-fos* RNA, maximal three to six days after the beginning of treatment.[27] It is of interest to notice that in T cell leukemias treatment with TPA, although inducing T cell maturation, rather decreased *c-fos* expression. Since normal T cells were reported to respond to TPA with increased *c-fos* transcription,[28] our data suggest that in leukemic T lymphocytes *c-fos* expression may be regulated differently.

The finding that in fresh human leukemic cells *c-fos* expression is restricted to a monocytoid phenotype in ANLL and to a subset of B cell leukemias that express the oncogene either constitutively or following activation by TPA could suggest different interpretations. A possibility is that it represents a phenomenon of "lineage infidelity",[29] in which a subset of leukemic B cells coexpresses B lymphocytic and monocytic markers. Alternatively, the restricted *c-fos* expression in human leukemias may mirror a stage of hematopoietic differentiation in which features of monocytic and B lymphocytic lineages are physiologically and transiently coexpressed and frozen in leukemic cells.[30] It is therefore tempting to speculate that the B lymphoid disorders expressing constitutively high levels of *c-fos*, ie, ALLs with the t(4,11) translocation, HCLs, and some B-CLLs, are those that have been recognized for a long time as showing a number of monocytic features (phagocytosis, enzyme activity, differentiation antigens) along with clear B cell markers (rearranged Ig genes, pan-B antigens, IL 2 receptor).[31-36] In conclusion, our results suggest that high constitutive levels of *c-fos* RNA may identify myelomonocytic oriented forms of leukemia in addition to particular subsets of B lymphoid malignancies and some cells terminally differentiated in vitro to a monocyte-like phenotype. In this view *c-fos* expression may be regarded as an additional marker for subclassification of human leukemias.

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Evidence for a Multipotential Stem Cell Disease in Some Childhood Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

By Naoki Tachibana, Susana C. Raimondi, Stephen J. Lauer, Peggy Sartain, and Lois W. Dow

Children with Philadelphia chromosome (Ph+) acute lymphoblastic leukemia (ALL) have a poorer prognosis than do most pediatric patients with ALL. Because of this poor prognosis and the presence of the Ph chromosome, we have asked whether or not Ph+ ALL involves a multipotential stem cell. We cultured hematopoietic progenitors from two children with Ph+ ALL and examined individual BFU-E and CFU-GM colonies for the Ph chromosome. We studied cells from two patients after 18 to 34 months of first complete clinical remission; direct cytogenetic analyses showed 26% and 13% Ph+ metaphases in these patients' marrow cells. BFU-E colonies were obtained from light density marrow cells cultured in methylcellulose supplemented with erythropoietin and CFU-GM colonies from agar or methylcellulose cultures stimulated with leukocyte feeder layers. Fifty-seven G-banded metaphases were recovered from 33 colonies. Ten metaphases from seven colonies were Ph+. Ph+ metaphases were found in three of 12 and three of five BFU-E colonies from the two patients. One of 16 CFU-GM colonies from one patient had the Ph+ chromosome; analyzable metaphases were not obtained from CFU-GM of the other patient. No colonies contained both Ph+ and Ph− cells. These results indicate that Ph+ ALL with persistence of Ph+ cells in remission involves a multipotential stem cell for erythroid and granulocyte/macrophage as well as lymphoid lineages. Multipotential stem cell involvement in the pathogenesis of some childhood Ph+ ALL suggests similarities to Ph+ chronic myelocytic leukemia and may contribute to the poor prognosis of these patients.

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ADVANCES in the treatment of acute lymphoblastic leukemia (ALL) have resulted in prolonged disease-free survival and cure in the majority of children with this disease.[1] However, some patients at high risk for treatment failure can be identified, including those whose blast cells contain the Philadelphia (Ph) chromosome.[2] Ph+ ALL and Ph+ chronic myelocytic leukemia (CML) share a common cytogenetic feature, but results of recent studies have demonstrated that these diseases may differ at the molecular level in alteration of the *bcr-abl* gene complex.[3-5] The persistence of the Ph chromosome in bone marrow cells from patients with Ph+ ALL in remission is a variable finding, whereas the Ph chromosome is generally found in marrow cells from patients with Ph+ CML in hematologic remission. Chromosomal abnormalities are generally absent from marrow cells from patients with Ph− ALL in remission. Previous studies of Ph− ALL have shown no evidence of nonlymphoid hematopoietic involvement in the leukemic process,[6] whereas Ph+ CML involves a multipotential stem cell for both lymphoid and myeloid cells.[7,8] To examine stem cell involvement in Ph+ ALL, we cultured bone marrow hematopoietic progenitor cells from such patients in complete clinical remission and examined mitoses from individual CFU-GM and BFU-E colonies for karyotypic evidence that might help to clarify the pathophysiology of this acute leukemia.

CASE REPORTS

Patient 1. This 4-year-old white boy presented at 12 months of age with pallor, fever, and hepatosplenomegaly. A complete blood cell (CBC) count disclosed hemoglobin 7.7 g/dL, leukocyte count of 38.8 × 10^9/L with 4% circulating blast cells, and platelet count of 41 × 10^9/L. A bone marrow aspirate showed 50% lymphoblasts, FAB-L$_1$. He had no mediastinal enlargement and CSF contained no blast cells. The marrow lymphoblasts were terminal transferase positive and had the pre-B cell phenotype with 63% of cells containing cytoplasmic immunoglobulin; the cells were positive for Ia, common acute lymphoblastic leukemia antigen (CALLA), BA-1, BA-2, Leu-16, and Leu-12 antigens. Cytogenetic studies showed five metaphases, all of which were positive for the t(9;22)(q34;q11) translocation.

Remission was induced after 4 weeks of four-drug induction chemotherapy after which he received 6 months of consolidation therapy with three rotating pairs of chemotherapeutic agents, followed by 24 months of therapy with 6-mercaptopurine and methotrexate.[9] CNS prophylaxis consisted of intrathecal methotrexate, hydrocortisone, and cytarabine. After 30 months of continuous complete remission (CCR), all therapy was stopped, and his disease has remained in CCR for an additional 8+ months. Marrow samples for in vitro studies were obtained between the 18th and 30th months of CCR.

Patient 2. This 7½-year-old white girl presented with recurrent fever, skin infections, abdominal and back pain, and hepatosplenomegaly. Laboratory studies showed anemia, thrombocytopenia, and a leukocyte count of 1,043 × 10^9/L with 46% blast cells, 2% bands, 1% metamyelocytes, 4% promyelocytes, 46% lymphocytes, and 1% monocytes. A bone marrow aspirate had 95% blast cells with a FAB-L$_2$ morphology. Spinal fluid contained 26 WBCs/mm^3 with 51% blast cells. There was no radiographic evidence of mediastinal adenopathy. The bone marrow blast cells were CALLA+ and T-3+ but negative for E rosette formation, other T cell markers, and surface and cytoplasmic immunoglobulin. Cytogenetic studies of marrow cells disclosed that all 18 metaphases analyzed were Ph+;

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seven metaphases had the 46,XX,t(9;22) karyotype, and 11 metaphases showed a side line: 45,XX,−8,−E,+mar,1q−,t(9;22). The marker appears to be a derivative eight chromosome with parts of an E group chromosome and the majority of the 1q arm translocated in tandem onto the 8q. Initial therapy consisted of leukopheresis and remission-induction chemotherapy with prednisone plus vincristine and intrathecal methotrexate, cytarabine, and hydrocortisone. Because her bone marrow still contained lymphoblasts after 4 weeks of therapy, two courses of cytarabine and daunorubicin were given, and remission was induced 3 months after diagnosis. Consolidation therapy included L-asparaginase, cranial irradiation, and intrathecal methotrexate followed by continuation therapy for 30 months with pulses of adriamycin, vincristine, cytarabine, and prednisone as well as 6-MP and methotrexate. All therapy was discontinued after 30 months of CCR. Five months later she suffered a hematologic relapse. A second remission was induced and was maintained for seven months. An attempt to induce a third remission failed, and she died 47 months after diagnosis. The cell sample for in vitro study was obtained 4 months after the cessation of therapy while the patient was in first remission.

MATERIALS AND METHODS

Cell culture. Light density (<1.077 g/cc) bone marrow mononuclear cells were obtained by separation on Ficoll-Hypaque (FH) gradients. For CFU-GM assays, cells were cultured over leukocyte feeder layers in 0.3% agar or in 0.8% methylcellulose at 1×10^5 cells/mL as previously described.[10] For BFU-E, cells were incubated in 0.8% methylcellulose at 5×10^4 cells/mL with 10% leukocyte-conditioned medium and 5 units of erythropoietin/mL.[11,12] CFU-GM are reported as colonies of >50 cells on day 10 and BFU-E as colonies of >50 hemoglobin-containing cells scored on day 14. The morphological classification of cells was confirmed by examining the cytology of individually picked colonies stained with Wright's stain or 3,3′-dimethoxybenzidine.[13]

Cytogenetic analysis. Direct bone marrow cytogenetic analysis was performed as previously described.[14] Marrow cells were collected in tubes containing 8 mL of RPMI 1640 with L-glutamine, antibiotics and 30% fetal calf serum (FCS). Cells were processed immediately after a 25-minute incubation in colcemid (final concentration, 0.06 μg/mL; Gibco, Chagrin Falls, OH), rinsed twice in Hanks' balanced salt solution (HBSS), and exposed to 0.075 mol/L KCl for 32 minutes at room temperature, including periods of mixing, standing, and centrifugation. The cells were then fixed in 3:1 methanol:acetic acid for 15 minutes. Slides were prepared by using the flaming-edging technique and were allowed to age for zero to seven days.

For analysis of individual colonies, day 8-to-day 14–cultured cells were incubated for one hour with colcemid (1 μg/mL) and colonies identified microscopically as to type. Individual colonies were transferred to microtiter wells for hypotonic lysis with 0.075 mol/L KCl for 20 minutes (BFU-E) or 30 minutes (CFU-GM) and then to polylysine-treated slides.[15] Chromosomes were allowed to adhere for ten minutes, after which the excess KCl solution was removed; 3:1 methanol:acetic acid fixative was added drop-wise and the preparations flame-dried. G-banding was performed by using a modification of the trypsin-Giemsa technique of Seabright.[16] Chromosomes were identified and assigned according to the International System of Human Cytogenetic Nomenclature.[17]

RESULTS

For patients 1 and 2, respectively, six and three bone marrow aspirates were obtained during remission in the period of the in vitro studies. These marrow cells were morphologically normal with <1% blast cells. In direct cytogenetic preparations of marrow cells from patient 1, 25 (26%) of 96 metaphases were Ph+, and for patient 2, 11 (13%) of 85 were Ph+. The remaining metaphases from each patient were normal.

In vitro cultures were attempted from three aspirates from patient 1 and one aspirate from patient 2. For patient 1 the number of CFU-GM colonies scored on day 10 ranged from 42 ± 2 to 290 ± 16 per 2×10^5 plated cells and the number of BFU-E colonies from 87 ± 3 to 117 ± 5 per 5×10^4 cells. For patient 2 the sample was obtained ten days before relapse,

Table 1. Cytogenetic Findings in Bone Marrow Colonies

Time of Study	Colony Type (culture condition)	Day of Culture	Karyotype
Patient 1			
17 mo CCR	CFU-GM (MC/F)	9	44,XY,−11,−18
	BFU-E	8	45,X,−Y
	BFU-E	8	46,XY,t(9;22)
	BFU-E	10	46,XY
	BFU-E	10	46,XY,t(9;22) × 2
24 mo CCR	CFU-GM (AG/F)	8	46, XY
	CFU-GM (AG/F)	10	46,XY
	CFU-GM (AG/F)	14	46,XY × 8
	CFU-GM (AG/F)	14	46,XY
	CFU-GM (AG/F)	14	46,XY × 4
30 mo CCR	CFU-GM (MC/F)	6	45,Y,−X
	CFU-GM (MC/F)	6	44,X,−Y,−22
	CFU-GM (MC/F)	6	45,X,−Y
	CFU-GM (MC/F)	6	46,XY
	CFU-GM (MC/F)	9	46,XY
	CFU-GM (AG/F)	10	45,XY,−10/45,XY,−20
	CFU-GM (AG/F)	10	46,XY
	CFU-GM (AG/F)	10	46,XY
	CFU-GM (AG/F)	10	46,XY
	CFU-GM (MC/EPO)	10	46,XY,t(9;22)
	BFU-E	9	46,XY/44,XY,−9,−21
	BFU-E	9	45,XY,−19, × 1/46,XY × 3
	BFU-E	9	46,XY
	BFU-E	9	46,XY × 2/45,XY,−14/45,XY,−22
	BFU-E	9	45,XY,−9,t(9;22)/46,XY,t(9;22)
	BFU-E	10	46,XY/45,XY,−9
	BFU-E	10	46,XY/44,XY,−19,−20
	BFU-E	10	45,XY,−22
Patient 2			
34 mo CCR	BFU-E	8	42,XX,−14,−15,−21,−21/41,XX,−17,−19,−20,−21,−22
	BFU-E	8	46,XX,t(9;22)/43,XX,−8,−11,−12,t(9;22)
	BFU-E	8	46,XX,t(9;22)
	BFU-E	8	45,X,−X,t(9;22)
	BFU-E	8	46,XX

and growth was low with 6 ± 1 CFU-GM per 1×10^5 in methylcellulose feeder-stimulated cultures and 11 ± 1 BFU-E per 5×10^4 cells.

Cytogenetic findings in bone marrow colonies are shown in Table 1. Fifty-seven well-banded analyzable chromosome spreads were available from 16 CFU-GM colonies and 17 BFU-E colonies. From one to eight analyzable metaphases were obtained per colony. Metaphases analyzed from one of 16 CFU-GM colonies and three of 12 BFU-E colonies from patient 1 and from three of five BFU-E colonies from patient 2 (21% of all colonies studied) had the t(9;22) translocation (Fig 1). The remaining 26 colonies studied lacked this translocation.

DISCUSSION

To our knowledge this is the first report with karyotypic evidence of erythroid and granulocyte/macrophage lineage involvement in ALL. Previous studies using G6PD isozymes[6] of childhood Ph− ALL have shown no evidence of leukemic involvement by nonlymphoid cells. Similarly, results of a study of erythroid colonies from one 48-year-old patient with ALL failed to demonstrate the presence of Ph chromosome.[18] In contrast, in Ph+ CML, involvement of multiple lineages, including erythroid, myeloid, megakaryocytic, and some lymphoid cells, have been well documented.[7,8]

The Ph chromosome has been identified in 5% to 7% of childhood ALL cases[2,19] and has been associated with a poor prognosis. Children with Ph+ ALL are more likely to be older, to have elevated leukocyte counts and CNS leukemia, and to be at higher risk of remission induction failure or earlier relapse.

The Ph chromosome was initially identified in 1960[20] and was subsequently shown by Rowley[21] to result from a reciprocal translocation involving chromosomes 9 and 22. Recent molecular biology studies have indicated that the Abelson leukemia virus oncogene homologue is translocated from chromosome 9 band q34 to chromosome 22 band q11, resulting in transcription of an Abelson-derived tyrosine-kinase messenger RNA and subsequent Mr 210 protein product.[22,23] Recently it has been reported that in Ph+ ALL patients, a different Abelson-derived tyrosine-kinase with a Mr of 185 or 180 is produced.[3-5] The Abelson-related protein kinase of cells from patient 1 has a Mr of 185 (S.S. Clark and O.N. Witte, personal communication). This molecular evidence and the clinical presentation indicate that this patient has Ph+ ALL, not CML in blast crisis.

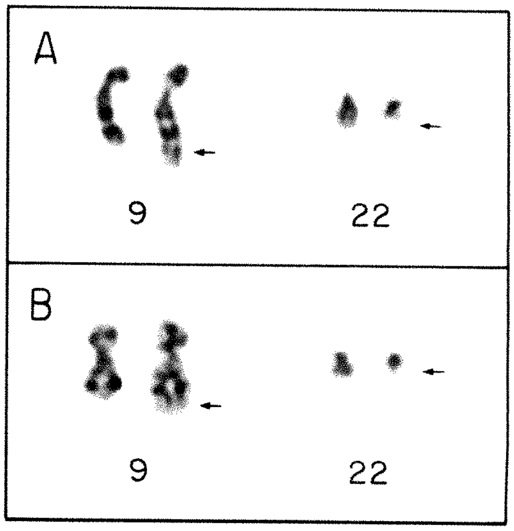

Fig 1. Partial G-banded karyotype of chromosomes involved in the t(9;22)(q34;q11) and their homologues. CFU-GM (A) and BFU-E (B) colonies from patient 1. Arrows indicate breakpoints.

In both of the patients we studied, Ph+ cells were present in their marrows during remission and appeared to be in stable mosaicism with karyotypically normal cells. Most patients who have Ph+ ALL at diagnosis have not had the Ph chromosome detected when their cells have been examined during disease remission.[2] Whether these patients have a different disease at the stem cell expression and molecular levels or an undetected mosaicism is unclear. Our study suggests that patients with Ph+ ALL may be heterogeneous and that further studies are indicated to determine lineage involvement compared to the molecular biology of these diseases.

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Iron Homeostasis in β-Thalassemic Mice

By David B. Van Wyck, Manuel E. Tancer, and Raymond A. Popp

To explore the pathogenesis of nontransfusional iron overload in iron-loading anemia, we examined features of external iron exchange, internal iron kinetics, and tissue iron burden in adult mice with inherited gene-deletion β-thalassemia. Mice homozygous for β-thalassemia display moderate anemia, reticulocytosis, and shortened red cell survival, whereas heterozygous carriers appear hematologically normal. Quantitative iron determination revealed that iron content and concentration in liver, spleen, and kidney, but not heart, were far higher ($P < .01$) in 15-to 35-week old homozygous thalassemic mice than in age-matched normal and heterozygous controls; of these tissues, iron content increased with age only in kidneys ($P = .01$) of homozygous affected mice. Although plasma iron levels were only minimally elevated in homozygotes, plasma iron turnover was threefold greater ($P < .001$) than that seen in heterozygote controls. Nevertheless hyperabsorption of enteric radioiron, discernible among homozygous thalassemic mice as late as 6 to 8 weeks after birth, was not observed in older mice; additionally, thalassemic and control mice at 18 to 34 weeks showed comparable iron excretion after intravenous radioiron. We conclude that adult mice with β-thalassemia regain balanced external iron exchange, despite substantial tissue iron excess and accelerated internal iron transit.

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IRON OVERLOAD is a poorly understood and potentially lethal manifestation of β-thalassemia. Patients suffering thalassemia major accumulate life-threatening iron overload after repeated red cell transfusions yet compound this transfusion-acquired iron excess with a pernicious and as yet unexplained avidity for iron contained in foods.[1] Less anemic but still troublesome[2] by their great numbers are patients with thalassemia trait, who without transfusions display evidence of iron overload in plasma iron, iron saturation, and ferritin levels measurably higher than normal.[3] Although increased gastrointestinal (GI) iron absorption and rapid internal iron turnover[4] have been demonstrated in patients with thalassemia minor,[5,6] further information about generation, maintenance, and extent of nontransfusional iron excess in carriers of the β-thalassemia gene is limited.

To explore mechanisms responsible for nontransfusional iron overload in iron-loading anemia, we examined iron balance and tissue iron levels in a recently developed model of inherited β-thalassemia in mice. The genetic defect in affected mice arises from an inheritable 3.7-kilobase (Kb) DNA deletion in mouse chromosome 7 encompassing both the regulatory and coding sequences of the gene for β-dmajor globin, a normal adult β-globin.[7,8]

Hematologic features of murine β-thalassemia depend on the number of defective genes inherited, degree of compensatory globin production, and resulting balance of α and β chain production. Among heterozygous mice bearing a single defective gene for β-dmajor globin, compensatory production of β-dminor[9] is complete, so that α and β chain synthesis remains balanced and phenotypic features normal. Among homozygotes, compensatory production of β-dminor globin is substantial but incomplete, so that synthesis of α and β chains is unbalanced (α:β synthesis ratio 1.7 in bone marrow, 1.3 in circulating reticulocytes). The associated short red-cell survival, modest anemia, microcytosis, poikilocytosis, reticulocytosis, and splenomegaly resemble β-thalassemia minor in humans.

METHODS

Experimental animals. Homozygous affected adult male mice, the offspring of homozygous breeding pairs back crossed onto a C57BL/6J background and third through fifth generation descendants of the proband male DBA/2 mouse in which a spontaneous deletion of the β-dmajor gene occurred,[7] served as test animals. For studies of GI iron absorption, groups of mice between 6 and 30 weeks old were selected; mice for all other studies were 15 to 35 weeks old. Normal C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice or heterozygous offspring of homozygote-by-C57BL/6J breeding pairs, matched for age, gender, and histocompatibility ($H\text{-}2^b$ antigen), sharing with test mice identical batches of standard chow, either Wayne Lab-Blox (iron approximately 340 μg/g; Allied Mills, Chicago) or Purina Lab Chow (iron approximately 380 μg/g; St Louis) ad libitum, served as controls. Heterozygote controls were chosen from offspring of homozygote-by-C57BL/6J pairs in preference to offspring of homozygote-by-heterozygote pairs because newborn homozygotes fail to compete successfully with nonhomozygous littermates. Similarly, mice older than 35 weeks were not examined because older homozygotes fail to thrive when compared to nonhomozygous mice of the same age. Clinical phenotype was confirmed as either normal or abnormal by hematocrit and red cell morphology, and hemoglobin genotype as normal, heterozygote, or homozygote by cellulose-acetate hemoglobin electrophoresis. After selection and matching, groups of experimental and control mice underwent studies of either plasma iron and iron-binding capacity, tissue iron levels, GI iron absorption, or plasma iron kinetics and excretion.

Blood sampling. Blood for plasma iron determination or radioiron studies was obtained by retro-orbital plexus phlebotomy using one heparinized 44.7 μL capillary pipette (Dade Diagnostics, Aguada, PR) per determination per mouse; blood for iron binding capacity was similarly obtained by filling two nonheparinized 150 μL capillary pipettes (Kimble, Toledo) per mouse; after phlebotomy, plasma was immediately separated from packed cells by centrifugation. Mice phlebotomized for iron binding capacity were excluded from further study.

Histology. Portions of tissue removed at sacrifice were examined by routine hematoxylin and eosin and trichome staining and hemosiderosis graded after Prussian blue staining.[10]

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Plasma iron. After a two-hour fast, experimental and control mice underwent phlebotomy between 9 and 10 AM, and plasma iron and iron binding capacity were determined by the coulometric method[11] on a FerroChem II analyzer (Environmental Sciences Associates, Bedford, MA).

Tissue iron. After killing by decapitation, liver, spleen, kidneys, lungs, and heart were removed, dried, weighed, digested by standard methods,[12] and total iron content determined on a Perkin Elmer Model 603 atomic absorption spectrometer (Perkin Elmer, Norwalk, CT). Results were expressed as organ iron concentration (μg/mg dry weight) and iron content (μg/organ).

Metabolic cages. Mice were placed singly in plastic Nalgene (Nalge Co, Rochester, NY) metabolic cages the day prior to radioiron studies; water and powdered lab chow were provided ad libitum; stool and urine were collected separately. Preliminary experiments with these cages confirmed that experimental and control groups consume food in equal amounts and that collection of stool and urine over a seven- to 30-day period, coupled with determination of residual carcass activity, yields recovery of greater than 95% of administered radioiron activity.

Iron (Fe59 reference dose) absorption. Mice placed in metabolic cages, fasted for 16 to 20 hours, and lightly anesthetized under methoxyflurane (Metafane, Pitman-Moore, Washington Crossing, NJ), received 0.1 μCi Fe59 administered intragastrically in 0.2 mL of a solution containing ferrous sulfate (10 μg/mL) and ascorbic acid (250 μg/mL), so that each dose provided 0.03 to 0.005 μg label iron, 0.4 μg (7 nmol/L) carrier iron and 50 μg (280 nmol/L) ascorbate.[13] Activity of stool and urine collected separately for seven days thereafter was counted in a Packard Auto-Gamma scintillation spectrometer (Packard Instruments, Downers Grove, IL) with channel settings of 1.0 to 1.2 meV and results expressed as percent total dose absorbed. Radioiron for all studies was obtained as Fe59Cl (New England Nuclear, Boston, MA), specific activity >6 μCi/μg, diluted to >40 μCi/mL in 0.5 M H_2SO_4.

Iron (Fe59) kinetics in plasma. In unanesthetized mice after a two-hour fast, radioiron (0.75 μCi Fe59 as ferrous sulfate, in 0.1 mL 5% dextrose and water, providing less than 0.1 μg total iron) was administered slowly over a three-minute period via tail vein injection. In preliminary experiments, electrophoresis on nondenaturing polyacrylamide gel of plasma obtained five minutes following radioiron infusion confirmed that detectable plasma radioactivity was confined to a single band corresponding to that of purified mouse transferrin (Cooper Biochemicals, Malvern, PA). Beginning five minutes after completion of Fe59 injection and concluding five to ten minutes after the first plasma radioiron T½, five serial samples of whole blood were obtained, plasma and red cells separated immediately, and sample radioactivity determined as above. Plasma iron levels were determined on blood obtained just prior to injection. Thus total volume of blood removed in six samples approximated 300 μL whole blood per mouse. From these results plasma radioiron disappearance and T½ were obtained by linear least squares regression and plasma iron turnover (PIT; μg/dL whole blood/day) calculated according to the following formula[14]:

$$\text{PIT} = \frac{\text{Plasma Iron } (\mu\text{g/dL})}{\text{T}1/2} \times \frac{100 - .9 \times \text{Hct}}{100}.$$

Mice from these studies were then examined for radioiron excretion.

Iron (Fe59) excretion. In mice housed singly in metabolic cages after IV Fe59 injection as above, urine and stool were collected for 30 days, radioactivity determined, and results expressed as percent excretion of total injected dose.

Statistical analysis. When experiments involved three groups, differences were examined by Duncan's multiple range T test; for two groups differences were examined by Student's *t* test. Correlations were performed and significance of relationships determined by a general linear hypothesis; each test was performed using a statistical applications package[15] on an IBM-PC/AT (International Business Machines, Boca Raton, FL) configured with an 80287 math coprocessor. Results from each set of studies, including those from absorption experiments, were found to be normally distributed.

RESULTS

Tissue iron. Tissue examination in 15- to 35-week old homozygous β-thalassemic mice revealed light (1+) iron staining in liver, scattered staining in renal tubules, and heavy staining in spleen and bone marrow, without evidence of tissue damage; splenomegaly but not hepatomegaly; and, after tissue iron determination, high iron concentration and total organ-iron content (Table 1) in liver, spleen, and kidneys. By contrast, organs from age-matched heterozygous β-thalassemic mice, like those from normal C57BL/6J controls, showed no parenchymal iron histologically and low organ iron levels. Although heart weight (and therefore iron content) was lower in normal than heterozygous controls, no other differences between these groups were found by multiple range T test. Only in kidneys from homozygous thalassemic mice was there a discernible relationship between age (15 to 35 weeks) and tissue iron concentration ($r = .615$, $P = .058$) or iron content ($r = .666$, $P = .01$). In kidneys from other groups and in other organs from all three groups, correlation between age and organ-iron concentration yielded r values no greater than .281 and between age and iron content no greater than .276.

Table 1. Organ Iron Distribution in Mice With β-Thalassemia Compared to Heterozygote and Normal Controls

Genotype	N	Age (wk)	Body Weight (g)	Dry Weight (mg)				Iron Concentration (μg/mg)				Iron Content (μg)				Total Content Four Organs
				Liver	Kidney	Heart	Spleen	Liver	Kidney	Heart	Spleen	Liver	Kidney	Heart	Spleen	
Homozygous	10	22.4	28.1	443.0	90.4	41.8	91.4	.795	.523	.552	5.800	350.5	47.2	22.6	522.3	942.5
β-thalassemia		4.8	2.1	46.8	16.9	6.4	17.4	.239	.145	.124	1.500	104.2	16.0	3.4	133.6	168.4
Heterozygous	6	18.6	27.2	346.3*	81.3	41.1†	25.9*	.265*	.286*	.609	1.188*	90.8*	23.5*	24.8†	27.0*	166.1*
β-thalassemia		1.5	3.1	55.9	33.0	5.7	17.2	.039	.040	.155	.645	14.1	11.1	5.9	13.4	25.2
Normal C57BL/6J	7	22.2	28.5	402.6	81.9	30.7*	15.7*	.181*	.220*	.513	1.510*	71.0*	17.1*	15.2*	24.4*	127.7*
		2.8	1.9	82.2	22.6	13.5	2.0	.061	.056	.064	.798	22.3	2.1	5.8	15.2	32.6

Results given as mean ± SD.

Differences (by Duncan's multiple range T test):

*Different from homozygotes by $P < .05$.

†Different from normal by $P < .05$.

Table 2. Plasma Iron and Iron-Binding Capacity in Homozygous β-Thalassemic Mice Compared With Heterozygous Controls

	N		Age (d)	Plasma Iron (μg/dL)	TIBC (μg/dL)	Saturation (%)
Homozygous β-thalassemia	10	mean	116.0	138.7	431.3	32.2
		± SD	0.0	13.4	13.0	3.2
Heterozygous control	10	mean	117.6	112.5	399.3	28.2
		± SD	2.2	18.6	16.9	4.7
P value				.002	.000	.040

Plasma iron and iron binding capacity. Homozygous-affected mice showed plasma iron, iron-binding capacity, and percent saturation levels slightly but significantly higher than those seen in age-matched unaffected heterozygote controls (Table 2).

Intestinal radioiron absorption. GI absorption of a low-dose Fe59 standard after a 16-hour fast was significantly greater in 6- to 8-week old β-thalassemic homozygotes than in age-matched heterozygote controls; however, as age advanced absorption among homozygotes declined, so that no differences were detected in older groups of mice (Fig 1).

Plasma radioiron kinetics. After a single IV Fe59 injection, β-thalassemic homozygotes showed more rapid plasma iron disappearance (Fig 2), initial red cell iron uptake (Fig 3), and plasma iron turnover (Table 3) than age-matched (range: 18 to 34 weeks) heterozygote controls.

Intestinal radioiron excretion. Total fecal excretion of Fe59 30 days after slow IV injection was no different in seven homozygotes for β-thalassemia than in six heterozygote controls (19.5% ± 3.8% *v* 24.6% ± 8.2% injected dose, mean ± SD). No significant accumulation of radioiron was detected in urine.

DISCUSSION

Our results confirm that β-thalassemic mice, like their human counterparts, acquire substantial nontransfusional iron excess: homozygous-affected mice show tissue iron levels up to fourfold higher than normal. Liver and spleen assume most of the increased iron burden, but kidneys also show higher than normal iron levels.

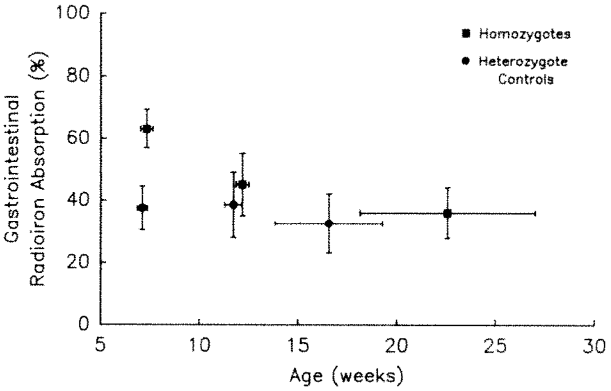

Fig 1. GI absorption of an Fe59 reference dose in homozygous thalassemic mice compared to heterozygote controls. Results represent mean ± 95% confidence intervals from age-selected groups of ten to 20 mice each. Hyperabsorption of iron was seen in homozygotes at 6 to 8 weeks of age but not in older mice.

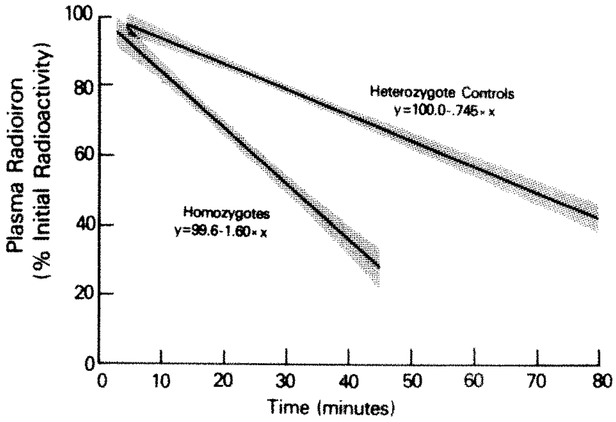

Fig 2. Plasma Fe59 disappearance early after slow IV injection in homozygous thalassemic mice compared to heterozygote controls. Results represent mean ± 95% confidence interval (shaded) from seven homozygotes and six heterozygotes, respectively; r values of individual clearance curves ranged from .96 to .99.

Despite accelerated internal iron transit, high internal iron stores, and early-occurring hyperabsorption of iron, homozygotes older than 11 to 13 weeks of age display normal GI iron absorption and fecal iron excretion and no progression of iron excess thereafter, evidence that following acquisition of iron overload early in life, adult thalassemic mice successfully regain iron homeostasis. These results suggest that spontaneous nontransfusional iron overload in thalassemia arises not from intrinsically flawed iron regulation but rather from the normal response of intact iron control mechanisms to the abnormal stimulus of increased erythropoietic activity.

The close resemblance of homozygous β-thalassemia in mice to heterozygous β-thalassemia in humans provides further evidence that the molecular origin of both the hematopoietic and iron-loading disorders is a discrete genetic defect generating unbalanced production of α and β-globin chains. Deficient production of β-dmajor chains alone fails to

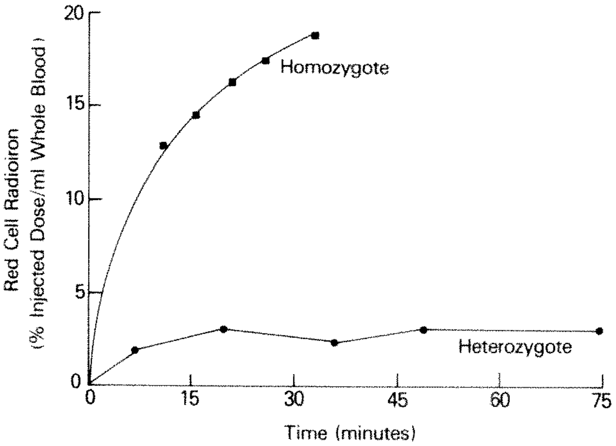

Fig 3. Representative Fe59 uptake in red cells early after IV injection in a homozygous β-thalassemic mouse compared to a heterozygous control. Within the first plasma half-life of Fe59, erythrocyte radioiron uptake was far more avid in homozygotes than in controls.

Table 3. Plasma Iron Kinetics in Homozygous β-Thalassemic Mice Compared to Heterozygous Controls

Genotype	N		Age (wk)	Body Weight (g)	Packed Cell Volume (%)	Plasma Iron Conc (µg/dL)	Plasma Iron T1/2 (min)	Plasma Iron Turnover (mg/dL whole blood/d)
Homozygote β-thalassemia	6	Mean ± SD	32.6	32.5	33.5	129.1	34.4	2.46
			5.4	3.3	3.3	35.4	5.5	.26
Heterozygote control	4	Mean ± SD	29.4	30.9	48.3	103.0	69.6	.77
			3.9	3.7	1.8	14.5	8.5	.08
P value			.340	.490	<.001	.206	<.001	<.001

account for all manifestations of the murine disorder, since heterozygous mice carry a single defective β-dmajor gene, produce lower than normal levels of β-dmajor globin, yet display red cell morphology and tissue iron levels indistinguishable from normal. Seemingly, by fully matching lost β-dmajor globin with compensatory β-dminor, heterozygotes are spared the consequences of β-dmajor gene deletion. In contrast, homozygotes, despite increased total hemoglobin production evidenced by rapid plasma iron turnover, fail to balance α and non-α globin synthesis. Whether iron loading in homozygous mice arises directly from unbalanced globin synthesis or indirectly from sustained acceleration of hematopoiesis cannot be determined from the current studies.

This relationship between phenotype and genotype facilitates experimental design: breeding heterozygotes from homozygotes on the one hand and normal mice on the other produces suitable unaffected controls for affected homozygotes, maintains a degree of genetic uniformity between experimental and control mice, yet avoids potential variation introduced by age, disease, diet, and transportation in commercially available mice.

Among tissues tested from homozygous thalassemic mice, only kidneys show a tendency to gain iron concentration and content with age. Although the relative contribution of renal iron to total body iron is low, age-related increase in renal iron may signify renal accumulation of filtered hemoglobin-bound iron after ongoing low-grade intravascular hemolysis.

Results of these studies in mice should be applied with caution to interpretation of the human disorder because iron homeostasis in small animals is distinguished from that in humans by greater potential for intestinal iron excretion,[16] greater dependence of hemoglobin synthesis on gut-absorbed rather than tissue-stored iron,[17] and exaggerated diurnal fluctuations in plasma iron levels according to feeding behavior. These considerations notwithstanding, the current results, taken together with our earlier experience in α-thalassemic mice,[18] suggest that examination of murine thalassemia may provide valuable insight into the pathogenesis of inborn errors of iron metabolism in man.

ACKNOWLEDGMENT

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Residual Juvenile Chronic Myelogenous Leukemia Cells Detected in Peripheral Blood During Clinical Remission

By Zeev Estrov, Ian D. Dubé, Helen S.L. Chan, and Melvin H. Freedman

At diagnosis peripheral blood (PB) or bone marrow from patients with juvenile chronic myelogenous leukemia (JCML) have shown two reproducible abnormalities when studied in cell culture: impaired growth of normal hematopoietic progenitors and excessive proliferation of malignant monocyte-macrophage elements. We used these findings to assess quality of treatment response by serially studying PB specimens from four JCML patients (patients 5, 7, 8, and 9) in complete chemotherapy-induced remission. PB readily yielded high numbers of monocyte-macrophage colonies in CFU-C and CFU-GEMM assays when cultured in early remission, and the colonies were cytogenetically proven to have arisen from a malignant clone in patient 9. When studied later in remission, the abnormal cell proliferation persisted in three of the four patients, but in patient 8 PB colony growth resembled controls. Similarly, when PB from patient 8 was studied in liquid culture without using added growth factor, cell proliferation declined identical to controls, whereas PB from the other three patients showed exuberant growth of monocyte-macrophage elements. Patient 8 successfully completed therapy and has been in a long-term, disease-free remission. The other three had recurrent, ultimately fatal disease. The cell cultures have allowed detection of residual abnormal cells that circulate in PB of JCML patients in remission. Although patient numbers were small because of the rarity of JCML, the data suggested that persistence of leukemia cells in these patients had a bearing on clinical outcome.

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JUVENILE chronic myelogenous leukemia (JCML) is a severe progressive hematopoietic malignant disorder of infants that represents less than 1% of all childhood leukemias.[1,2] Although the long-term survival of children with acute leukemia has significantly improved,[3,4] the median survival of JCML patients is still less than 10 months.[1,2] Recent clinical trials using aggressive chemotherapy protocols have increased the complete remission rates in JCML; however, few patients have achieved long-term, disease-free survival.[5-12]

An important observation that has become a hallmark of JCML is the abnormal in vitro growth characteristics of specimens obtained from newly diagnosed patients. When cultured in semisolid systems or in liquid cultures, bone marrow (BM) or peripheral blood (PB) mononuclear cells from patients with JCML yielded excessive numbers of monocyte-macrophage colonies in the presence or absence of added growth factor.[13-15] These cell culture findings have been reliable and reproducible and have become an essential diagnostic tool for JCML in our laboratory.

Since four of our recently diagnosed patients with JCML attained complete remission with chemotherapy, we used the cell culture findings to assess the quality of treatment response by serially studying their PB specimens. The data suggested that persistence of residual leukemia cells when in remission had a bearing on clinical outcome.

MATERIALS AND METHODS

Subjects. All four patients fit the classical clinical and laboratory description of JCML.[1,2] Their data at diagnosis were reported previously in *Blood*[15] and correspond to patients 5, 7, 8, and 9 in that series. They presented with varying combinations of lymphadenopathy, skin manifestations, variable splenomegaly, pallor, and hemorrhagic signs. Most had anemia, leukocytosis, monocytosis, thrombocytopenia, and nucleated RBCs in PB. BM aspirates were cellular with granulocytic hyperplasia, monocytosis, active erythropoiesis, and reduced-to-absent megakaryocytes. All patients were treated with combined chemotherapy used for acute nonlymphocytic leukemia[16] and were followed for 12 months to 3 years. Their chemotherapy was given as described[17] and consisted of varying combinations of daunomycin, cytosine arabinoside, 5-azacytidine, VP-16-213, 6-thioguanine, and 6-mercaptopurine. These studies were performed with informed consent and were approved by the Human Experimentation Committee of our institution.

CFU-C and CFU-GEMM assays and liquid cultures. Heparinized PB cells from the four patients and PB from five healthy volunteers used as controls were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 × g, 4°C) for 20 minutes to remove neutrophils and red cells.

The CFU-C assay was performed in methylcellulose as previously described[18] using 20% colony stimulating activity (CSA) prepared from peripheral blood leukocytes of a normal volunteer.[19] Colonies were counted after 14 days using an inverted microscope. A CFU-C colony was defined as a cluster of 20 or more cells consisting of granulocytes, monocyte-macrophages, or both. Microscopically, pure granulocytic colonies could be easily distinguished from pure macrophage colonies because of smaller cells and tighter clustering in the former.

For the liquid cultures, nonadherent PB mononuclear cells (5×10^6 cells/mL) were incubated in α-medium containing 10% fetal calf serum (FCS) for ten days as previously described.[20]

The CFU-GEMM assay was performed in methylcellulose[21,22] using 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA-LCM),[23] human erythropoietin 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver) or sheep erythropoietin 2.0 U/mL (Connaught Laboratories, Willowdale, Ontario), and 5×10^{-5} mol/L of 2-mercaptoethanol. All cultures were evaluated after 14 days for the number of BFU-E colonies, defined as an aggregate of greater than 500 hemoglobinized cells or three or more erythroid subcolonies, CFU-C colonies of granulocytic or macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a

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micropipette and were analyzed for cellular composition and karyotype.

Cell characterization and cytogenetic studies. Individual colonies were plucked from cultures with a micropipette, spread on glass slides, and the cellular composition was determined microscopically using Wright's stain and nonspecific (alpha naphthyl butyrate) esterase staining with and without treatment with fluoride.[24]

The cell surface antigens of individual CFU-C colonies were assessed using the following monoclonal antibodies (MoAbs) with intestinal alkaline phosphatase as previously described[25-27]: OKIa (Ortho Diagnostic Systems Inc, Raritan, NJ) that detects HLA-D framework antigen; Leu-M3, OKM1 (Becton-Dickinson, Mountain View, CA) that identifies monocytes and macrophages; Leu-1 and Leu-4 and B1 and B4 (Becton-Dickinson) that detect T and B cells respectively. The slides were counterstained in hematoxylin, and the presence of surface antigen was indicated as bright red granular cellular inclusions seen by light microscopy. Morphological details were well preserved, and a very high degree of correlation between morphology and surface antigen expression was observed. The intensity of staining was defined as strong (+ + +), moderate (+ +), and weak (+).

BM and PHA-stimulated PB mononuclear cells were cultured for 24 and 72 hours respectively and were then harvested for cytogenetic studies by routine procedures.[28] Individual JCML CFU-C colonies were plucked for cytogenetic analysis according to published methods.[29]

Statistical analysis. The probability of significant differences between colony numbers was determined by Student's t test.

RESULTS

The four patients attained complete clinical, hematologic, and BM remission following one to four chemotherapy courses.[17] During remission all signs and symptoms of JCML disappeared, and blood counts and BM aspirates were normal.

Patient 5 was given 21 courses of chemotherapy; the remission lasted 21 months but was followed by acute myeloblastic leukemia. Patient 7 received 16 courses of chemotherapy; the remission lasted 21 months but was followed by acute myelomonocytic leukemia. Patient 9 received 9 courses of chemotherapy; JCML recurred after 11 months. Patients 5, 7, and 9 have subsequently died of progressive disease. Patient 8 received 16 courses of chemotherapy over an 18-month period, after which treatment was stopped; at the time of writing patient 8 has been in complete remission for 15 months.

PB mononuclear cells from the four patients were studied at diagnosis and serially during remission using the CFU-C assay (Table 1). At diagnosis there was excessive proliferation of CFU-C colonies compared to the controls ($P < .005$). Characterization studies of individual colonies by morphology and cytochemistry confirmed that they were comprised of monocyte-macrophage elements; 85% of the cells stained intensely for nonspecific esterase and the stain was inhibited by fluoride. Immunocytochemically, 90% of cells in JCML colonies were positive for OKIa, greater than 90% were positive for Leu-M3, and greater than 80% expressed OKM1. All cells were negative for Leu-1, Leu-4, B1 and B4.

Cultures were performed several times during remission in the four patients when WBC numbers and differential counts were normal. Representative results are shown in Table 1 at two time periods, in early clinical remission, and later while on long-term chemotherapy. Initially in early remission colony numbers were increased compared to control values, except for patient 7, and the cellular composition of the CFU-C colonies from all four was exclusively of monocyte-macrophage lineage. The cells had the identical morphological, cytochemical, and cell surface phenotype as the cells harvested from colonies at diagnosis. In contrast, control CFU-C colonies were about 50% granulocytic, 40% monocyte-macrophage, and 10% mixed lineage.

Table 1. Peripheral Blood CFU-C Colony Proliferation at Diagnosis and Serially During Clinical Remission

	CFU-C Colonies/2 × 10^5 Cells Plated				
Patient ID No.*	5	7	8	9	Controls
At diagnosis	>1000	>1000	224	735	63 ± 11
Early remission†	320	70	135	218	—
Late remission‡	170	37	70	112	—
Remission, 7 mo off therapy	—	—	18	—	—

Data shown are mean colony numbers from duplicate cultures.

*See reference 15.

†Patients no. 5, 7, 8, and 9 were studied after 5, 4, 4, and 3 months of chemotherapy, respectively.

‡Patients no. 5, 7, 8, and 9 were studied after 20, 14, 16, and 15 months of chemotherapy, respectively.

Later in remission, while receiving chemotherapy, colony numbers were decreased or comparable to controls in patients 7 and 8 respectively but still increased in patients 5 and 9. The cellular composition of the colonies in patients 5, 7, and 9 still showed exclusive proliferation of monocyte-macrophage colonies. Results from patient 8 were different. At the conclusion of 16 months of chemotherapy, the CFU-C colonies had changed in composition and consisted of about 50% granulocytic lineage, identical to controls. Chemotherapy was stopped for patient 8 because the treatment protocol was completed, and the patient was retested seven months later while still in remission (Table 1). Colonies were somewhat reduced in number but still consisted of a normal pattern of about 50% granulocytic, 40% monocyte-macrophage, and 10% mixed lineage.

When studied at diagnosis in liquid culture without added CSA, PB cells from the four patients showed a high cellular proliferative rate compared to control PB cells. After ten days in culture, patients' cells increased numerically by approximately 70% to 150%, whereas control cells decreased by about 10%. A differential count of nonadherent JCML cells obtained on day 10 consisted of 13% monoblasts, 32% early monocytes, 54% mature monocytes, and 1% premature and fully differentiated macrophages. In the control cultures the nonadherent cells were comprised primarily of granulocytic and lymphocytic-appearing elements and a small population (less than 5%) of mature monocytes and macrophages. The majority of monocyte-macrophage cells in the control cultures were found in the adherent layer.

In remission, PB cells from patients 5, 7, and 9 still showed the abnormal, excessive proliferation of monocyte-macrophage elements in liquid culture even though routine peripheral blood cell numbers and differential counts were unremarkable. Results from patient 8 were different. After ten days of culture cell numbers decreased, and most cells became adherent to the plastic culture flasks, similar to controls.

In the CFU-GEMM assay, PB cells from the four patients at diagnosis yielded large numbers of monocyte-macrophage CFU-C colonies but absent BFU-E and mixed colony formation (Table 2). During early remission CFU-C colonies declined in number compared to the initial studies, and BFU-E and mixed colonies were detected.[15] However, CFU-C colonies in all four patients consisted of more than 90% monocyte-macrophage cells with an obvious background of individual monocytes and macrophages in the culture dishes. Cytogenetic analysis (17 metaphases) of CFU-C colonies from patient 9 showed the identical, abnormal karyotype (45,X,−Y) that was demonstrated in the diagnostic marrow, whereas concurrent analysis of PB lymphocytes showed a normal male karyotype (46,XY). Patient 5 was studied several months later while still in remission and on chemotherapy, and the CFU-C colony growth was increased and exclusively of monocyte-macrophage lineage (Table 2). Patient 8 was studied in remission 7 months after stopping chemotherapy. CFU-C numbers were decreased, and the cellular composition was approximately 50% granulocytic, similar to controls.

DISCUSSION

The unusual growth behavior of BM and PB JCML cells in vitro has facilitated characterization studies of this syndrome. In our recent study, the major finding in cell cultures of JCML BM and PB was an excessively high number of CFU-C colonies in CFU-C and CFU-GEMM culture assay systems.[15] These CFU-C colonies were comprised exclusively of monocyte-macrophage elements, as confirmed by morphology, cytochemistry, and surface marker studies, and were proven to arise from a malignant clone by cytogenetic analysis of individual colonies. The growth requirements for JCML CFU-C were also atypical because added CSA and the presence of adherent cells were not essential for JCML CFU-C to proliferate into colonies. In liquid culture JCML cells showed similar, abnormal growth characteristics and proliferated rapidly and excessively in the absence of an added humoral growth factor.[20] The clonal expansion of the malignant cells in liquid culture allowed us to perform detailed analysis of cellular lineage, functional properties, and potential for cellular differentiation.[20]

Table 2. Peripheral Blood CFU-GEMM Assay at Diagnosis and During Remission

	Colonies/Culture					
	At Diagnosis			During Clinical Remission†		
Patient ID No.*	CFU-C	Mixed	BFU-E	CFU-C	Mixed	BFU-E
5	>1000	0	0	45	4	80
				170	1	14
7	>1000	0	0	85	5	50
8	189	0	0	58	3	82
				26	0	12
9	137	0	0	90	6	190
5 controls	90 ± 10	8 ± 3	130 ± 12			

Data shown are mean colony numbers from duplicate cultures.

*See reference 15.

†Patient no. 5 was studied twice after 12 and 21 months of chemotherapy (upper and lower values, respectively). Patients no. 7 and 9 were studied after 10 and 13 months of chemotherapy, respectively. Patient no. 8 was studied after 16 months of chemotherapy and after being off therapy for 7 months (upper and lower values, respectively).

Thus assays for quantitation of JCML cells have become established and validated. The cell cultures have been of central importance in our laboratory for making a definitive diagnosis of this disease in new cases presenting as difficult diagnostic problems. In the present study the objective was to determine whether the assays were sensitive enough to detect small numbers of residual JCML cells from patients in chemotherapy-induced complete remissions, similar to our findings in acute lymphoblastic leukemia.[30]

The four JCML patients who were studied were all clinically and hematologically normal and hence in "complete remission." Careful inspection of PB white cell morphology by detailed differential counting did not disclose abnormal cells. Yet in all four patients studied in early remission and in three patients studied later in the treatment course, the in vitro findings were clearly abnormal in the clonogenic assays and in liquid cultures and confirmed persistent, disordered hematopoiesis. Even in studies in which colony numbers were in control range, the cellular composition of the colonies was strikingly aberrant, being exclusively of monocyte-macrophage lineage. The findings in patient 8 were different because the initially abnormal growth pattern became identical to controls with therapy. This change in the in vitro findings was clear-cut and was readily identified by several observers in our laboratory when the cultures were examined "blindly."

When tissue cultures are used to study leukemia cell proliferation, it is ideal to confirm the leukemic origin of cultured cells by demonstrating clonality. This may be accomplished by using cytogenetic markers, glucose 6-phosphate dehydrogenase isoenzymes in appropriate females, and by molecular approaches. Fortunately, JCML CFU-C from one of our patients showed a chromosomal abnormality that was previously described in this disorder.[31] Thus we were able to prove that the patient's PB clonogenic CFU-C that were detected during remission were of leukemic origin. In the other cases our data indicate that the unusual growth behavior of JCML cells in vitro can be successfully utilized to detect residual disease. This is especially important in JCML patients who do not show a marrow cell cytogenetic abnormality at diagnosis.

Thus these assays allowed identification of abnormal cells that were circulating in PB of our JCML patients in remission. An obvious question that arises is whether the finding of persistent JCML cells had prognostic significance. The answer is difficult because JCML is rare, and large numbers of patients are not available for study. However, of our four cases, the three that showed evidence of persistent, residual JCML had recurrent, fatal disease. The other case, in whom

JCML colony-forming cells were no longer detected, completed the therapy protocol and at the time of writing has been in long-term complete remission, off treatment.

The data indicate that persistence of leukemia cells in our patients had a bearing on clinical outcome. Cell cultures should be useful, therefore, in assessing the degree of efficacy of treatment programs that are designed to eradicate the disease.

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The β-Globin Gene on the Chinese $\delta\beta$-Thalassemia Chromosome Carries a Promoter Mutation

By George F. Atweh, Xiao-Xiang Zhu, Howard E. Brickner, Carol H. Dowling, Haig H. Kazazian Jr, and Bernard G. Forget

A new type of $\delta\beta$-thalassemia characterized by decreased expression of the β-globin gene and increased expression of both $^G\gamma$ and $^A\gamma$ globin gene in the absence of a detectable deletion has recently been described in the Chinese population. In this study we characterize the mutant β-globin gene from this $\delta\beta$-thalassemia chromosome. An A to G transversion is identified in the "ATA" sequence of the promoter region that leads to decreased expression of the β-globin gene in vivo and in vitro. We also demonstrate the presence of this mutation in every individual with a high fetal hemoglobin phenotype in this family and its absence in every individual with a normal hemoglobin phenotype. This same promoter mutation has recently been detected in Chinese β-thalassemia genes where it is present on chromosomes of the same haplotype as that of the $\delta\beta$-thalassemia chromosome we are studying. These data support the hypothesis that an as yet unidentified mutation occurred on the ancestral chromosome carrying the promoter mutation and subsequently gave rise to the $\delta\beta$-thalassemia phenotype.

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THE STUDY of the molecular pathology of the different thalassemic disorders over the last decade has uncovered an extensive array of mutations that lead to aberrant expression of one or more of the globin genes. The vast majority of the β-thalassemias have been associated with nucleotide substitutions, insertions, or deletions at regions within the β-globin gene that are of critical importance for the processes of transcription, splicing, polyadenylation, or translation of its mRNA.[1] The majority of the $\delta\beta$-thalassemias, on the other hand, are associated with extensive deletions or rearrangements within the β-globin gene cluster, which typically remove parts or all of the δ and β-globin genes along with variable amounts of flanking DNA. These deletions result in the activation of the remaining fetal globin gene(s).[1] We have recently described a new type of $\delta\beta$-thalassemia in a Chinese family characterized by decreased production of β-globin chains along with increased production of both $^G\gamma$ and $^A\gamma$ chains.[2] In the simple heterozygous state the affected individual's hemoglobin consisted of 22.3% hemoglobin F, which was heterocellularly distributed in the red cells. When this $\delta\beta$-thalassemia determinant was inherited with a β-thalassemia gene in a compound heterozygous state, the affected individual had the clinical picture of thalassemia intermedia, and his hemoglobin consisted of 15% Hb A (α_2, β_2), 3.6% Hb A_2 (α_2, δ_2), and 81.4% Hb F (α_2, γ_2). Initial gene mapping studies did not reveal any detectable deletion or rearrangement in the β-globin gene cluster. In this communication we report the structural and functional characterization of the β-globin gene on the $\delta\beta$-thalassemia chromosome of one of the individuals from this Chinese family. DNA sequence analysis of the cloned β-globin gene revealed a promoter mutation that is associated with a moderate decrease in β-globin mRNA production in a transient expression assay. We also utilized the technique of oligonucleotide hybridization to demonstrate the presence of this promoter mutation in every family member with the $\delta\beta$-thalassemia phenotype and its absence in those with a normal hemoglobin phenotype.

MATERIALS AND METHODS

β-Globin gene cloning and DNA sequence analysis. High mol wt DNA was isolated from the spleen of the compound heterozygous individual and from peripheral leukocytes of other family members using the method of Blin and Stafford.[3] The β-globin genes of the compound heterozygote were cloned as 7.5 kb *Hind III* fragments as described earlier.[4] The β-globin gene from the $\delta\beta$-thalassemia chromosome was identified by the presence of the *Ava II* restriction site in β-IVS-2 (Fig 1). This β-globin gene was subcloned as a 3.7 kb *Pst I/Bgl II* fragment in the expression vector πSVplac.[5] DNA sequence analysis was performed by the dideoxy chain termination method of Sanger et al.[6] The region of the cloned gene that was sequenced extends from 150 base pair (bp) upstream from the cap site to 200 bp downstream from the termination site, excluding 500 bp in the middle of IVS-2.

Gene expression studies. πSVplac recombinants containing the cloned mutant β-globin gene described above or a normal β-globin gene were cotransfected along with an α-globin gene containing recombinant into HeLa cells at 25% confluency using the technique of DNA coprecipitation with calcium phosphate followed by glycerol shock, as described earlier.[4] After 36 hours the cells were harvested, and cytoplasmic RNA was extracted using methods described by Favalaro et al.[7] The extracted RNA was used in S1 nuclease mapping experiments[8,9] with either a 3′ end-labeled, double-stranded probe or a uniformly labeled, single-stranded probe (see "Results" for description of specific probes). After S1 nuclease digestion, the protected DNA fragments were fractionated by electrophoresis in 6% or 8% polyacrylamide gels in the presence of 7 mol/L urea.

Oligonucleotide hybridization studies. Oligonucleotides (19 mer) specific for the −29 mutation and the normal "ATA" box region 5′ to the β-globin gene were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA). Ten microgram samples of genomic DNA of various subjects were subjected to oligonucleotide hybridization analysis as previously described.[10,11]

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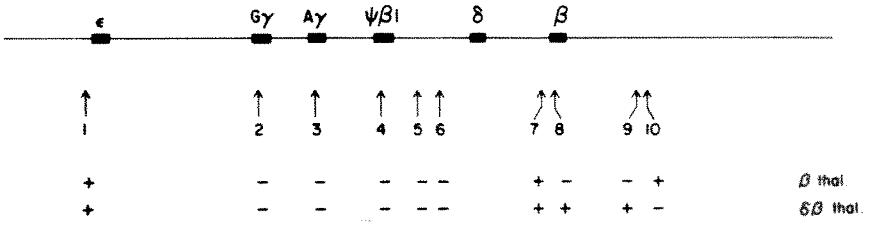

Fig 1. A map of the β-globin gene cluster. The numbered arrows represent the positions of polymorphic sites that were identified by the following restriction endonucleases: 1, 4, and 5, *Hinc II*; 2, 3, and 9, *Hind III*; 6, *Taq I*; 7, *Rsa I*; 8, *Ava II*; and 10, *Bam HI*. Abbreviations: (+), cleavage; (−), absence of cleavage at a site.

RESULTS

When the DNA sequence of the cloned β-globin gene from the δβ-thalassemia chromosome was obtained and compared to the sequence of a normal β-globin gene,[12] a single nucleotide difference was detected (an A to G transversion at position −29, Fig 2). This substitution occurs within the highly conserved "ATA" box sequence in the promoter region. This same promoter mutation is the commonest β-thalassemia mutation in American blacks, where it occurs on three chromosomal haplotypes that are all different from the chromosomal haplotype of this δβ-thalassemia chromosome.[13] More recently the same mutation has been detected in three Chinese β-thalassemia genes on a chromosomal haplotype identical to the haplotype of the patient under study.[14]

The mutant β-globin gene described above and a normal β-globin gene were transfected into HeLa cells, and the cytoplasmic RNA was isolated and subjected to S1 nuclease mapping studies. When a 3′ end-labeled probe that extends from the *Nco I* restriction site at the initiation codon to the *Bam HI* restriction site of exon 2 was used (Fig 3), the same 90-nt fragment was detected in both RNAs, which corresponds to a normally spliced exon 1. However, the globin-specific mRNA accumulated in the cells transfected with the mutant gene was 85% less than that accumulated in the cells transfected with a normal β-globin gene, after correction for differences in the efficiency of transfection by normalizing to the level of expression of the cotransfected α-globin genes (data not shown). These results are in agreement with those of Antonarakis et al[13] who studied an identical mutant gene isolated from an American black patient. We also did not detect any mRNA splicing abnormalities when we used a

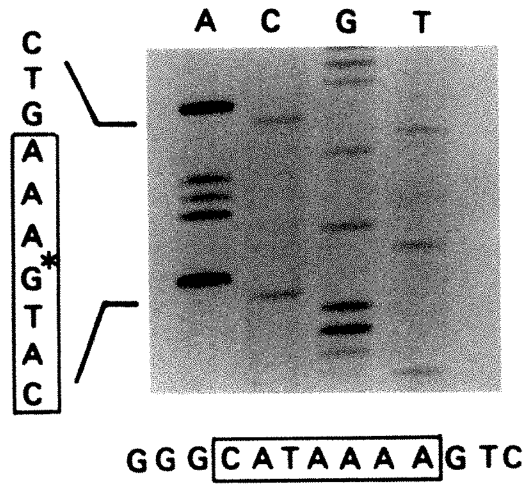

Fig 2. Autoradiograph of a DNA sequencing gel in the promoter region of the cloned β-globin gene. The normal DNA sequence is shown below the autoradiograph with the "ATA" box enclosed within the rectangle. The asterisk marks the A to G mutation within the ATA box.

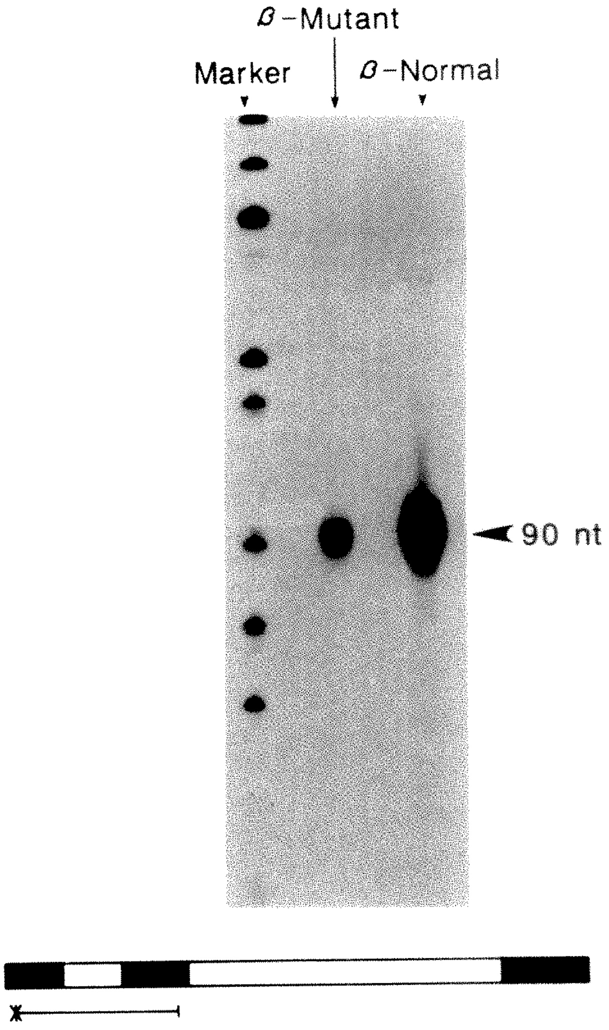

Fig 3. S1 nuclease mapping study of RNA transcribed from the β-mutant gene and a β-normal gene using a 3′ end-labeled probe represented on the schematic map below the autoradiograph. Both RNAs protect the same 90-nt fragment corresponding to exon 1. The amount of steady-state β-globin mRNA in the cells transfected with the mutant gene is 15% that of the cells transfected with the normal gene after correcting for differences in transfection efficiencies by normalizing to the level of expression of the cotransfected α-globin genes.

uniformly labeled, single-stranded probe that spans the four intron-exon junctions[4] (data not shown).

Finally, we studied the pattern of inheritance of this mutant gene in the extended Chinese family. A family pedigree is shown in Fig 4. Four of the eight family members who were available for study had a $\delta\beta$-thalassemic phenotype. Oligonucleotide hybridization analysis demonstrated that all of them had inherited the -29 promoter mutation in the β-globin gene (Fig 4). The remaining four members who did not have a $\delta\beta$-thalassemic phenotype did not inherit this mutant gene. These data provide strong evidence for the concordance of the high fetal hemoglobin phenotype and the promoter mutation in this family.

DISCUSSION

The observation that elevated levels of fetal hemoglobin seem to ameliorate the clinical severity of sickle cell anemia and homozygous β-thalassemia has sparked a great deal of interest in the study of the factors that regulate the production of fetal hemoglobin. Hereditary persistance of fetal hemoglobin (HPFH) and $\delta\beta$-thalassemia have served as prototypes of conditions in which the expression of the fetal globin genes persists at an elevated level into adult life. When the organization of the β-globin gene cluster of many individuals with these disorders was studied by gene mapping, it became obvious that numerous extensive deletions in and around the β-globin complex account for the majority of these cases. In spite of repeated efforts, the accurate mapping of these deletions has failed to convincingly demonstrate the presence of regulatory regions whose deletion may account for the increased expression of the fetal globin genes. The mechanism responsible for the activation of the fetal globin genes in those deletion disorders remains largely unknown, and different mechanisms may be operative in different types of deletion HPFHs and $\delta\beta$-thalassemias.

More recently, a few examples of HPFH that are not associated with a deletion in the β-globin cluster have become the focus of investigation. These disorders are characterized by the increased expression of either the $^{G}\gamma$ gene or the $^{A}\gamma$ gene but never both at the same time. Molecular studies of a number of these chromosomes have identified several interesting mutations in the promoter regions of either the $^{G}\gamma$ or $^{A}\gamma$ gene[15-19] that seem to be tightly linked to the HPFH phenotype.[11,20] It has been hypothesized that these mutations may be responsible for the increased expression of one or the other of the γ genes, either by increasing the binding affinity of a positive regulatory factor to the promoter region or by decreasing the binding affinity of a negative regulatory factor. Experimental support for either of these hypotheses has not yet been provided. Nonetheless these studies have provided important insight into the role of certain upstream promoter regions in the regulation of gene expression in eukaryotic cells. A single example of a nondeletion $\delta\beta$-thalassemia has been described in Sardinians where it is associated with an increase in the production of the $^{A}\gamma$-globin chains.[21,22] Molecular studies of this chromosome have demonstrated the presence of two independent mutations on the $\delta\beta$-thalassemic chromosome: a nonsense mutation at codon 39 of the β-globin gene and a promoter mutation at position -196 of the $^{A}\gamma$ gene.[23-25] When this same promoter mutation at position -196 occurs as a single mutation in Chinese or Italian individuals, the phenotype is that of $^{A}\gamma$ HPFH.[18,19]

The $\delta\beta$-thalassemia chromosome in our Chinese family is particularly interesting, since it is the only example of a nondeletion type of $\delta\beta$-thalassemia (or HPFH) where both $^{G}\gamma$ and $^{A}\gamma$ gene expression are markedly increased. The heterocellular distribution of fetal hemoglobin in simple heterozygotes and the clinical picture of thalassemia intermedia in compound heterozygotes for this determinant plus β-thalassemia have led us to classify this condition as $\delta\beta$-thalassemia rather than HPFH. We have determined that the interacting β-thalassemia gene in this family contains a previously undescribed nonsense mutation (manuscript in preparation). Since the compound heterozygote for this $\delta\beta$-thalassemia and β^0-thalassemia has 15% Hb A in his peripheral blood in the untransfused state, we conclude that the β-globin gene on the $\delta\beta$-thalassemic chromosome is active in vivo, albeit at a decreased rate.

Molecular cloning, DNA sequence analysis, and expres-

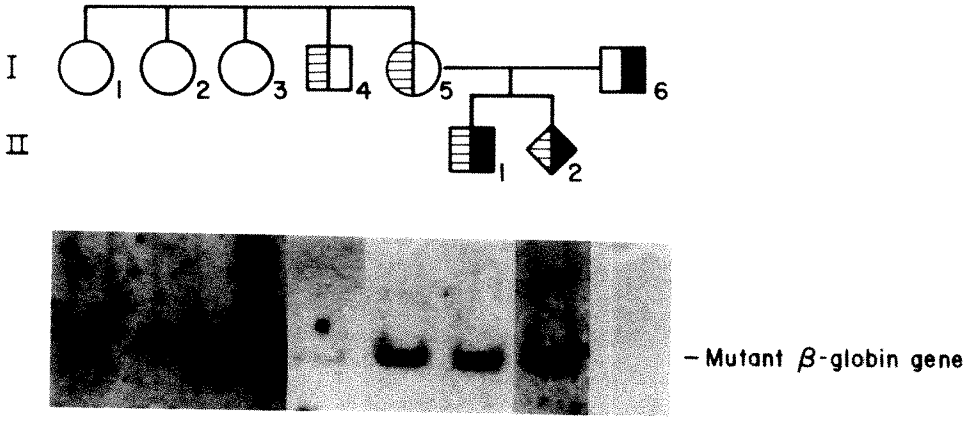

Fig 4. A pedigree of the affected Chinese family in which heterozygosity for β-thalassemia is denoted by solid black and $\delta\beta$-thalassemia by hatched circles and squares, respectively. The autoradiograph under the pedigree shows hybridization of an oligonucleotide probe specific for the -29 mutation to DNA from the different family members. Individuals I-4, I-5, II-1, and II-2 are positive for the tested mutant allele, while the remaining four individuals are negative.

sion studies of the β-globin gene on the $\delta\beta$-thalassemic chromosome identified a promoter mutation in the "ATA" box region that results in decreased expression of that gene in vitro. Study of eight individuals from this family by oligonucleotide hybridization showed a 100% concordance between this mutation and the $\delta\beta$-thalassemic phenotype. However, this does not imply that the -29 mutation is responsible for the increased expression of fetal globin genes in this situation, since the same mutation in American blacks and Chinese families is not associated with elevated levels of fetal hemoglobin. It merely provides evidence for strong linkage between the high fetal hemoglobin phenotype and the chromosome that bears this mutant β-globin gene.

We do not yet know the mechanism(s) responsible for the increased expression of fetal hemoglobin in this type of $\delta\beta$-thalassemia. As stated above, our data support the hypothesis that the mutation responsible for the high fetal hemoglobin phenotype is present in or near the β-globin gene complex. It is conceivable that two different mutations that activate the $^{G}\gamma$ and $^{A}\gamma$ genes independently may be present on the same chromosome that carries the β-globin gene with the promoter mutation. However, there is no precedent for the occurrence of three independent mutations on the same chromosome within a single gene family. Nevertheless we did rule out the presence of the -196 $^{A}\gamma$ C to T substitution[25] by oligonucleotide hybridization and that of the -202 $^{G}\gamma$ mutation[20] and -158 $^{G}\gamma$ mutation[26] by Southern blotting using the enzymes *Apa I* and *Xmn I* (data not shown). A more intriguing possibility is the occurrence of a single mutation in a regulatory region that affects the expression of both fetal genes and leads to the overexpression of the $^{G}\gamma$ and $^{A}\gamma$ genes. We also cannot exclude the possibility that a mutation in the δ-globin gene, with or without mutations in the fetal genes, may be responsible for the phenotype. The fact that the haplotypes of this $\delta\beta$-thalassemia chromosome and the β-thalassemia chromosome with the same "ATA" box mutation in the Chinese are identical supports the hypothesis that the mutation(s) responsible for the increased expression of the γ-globin genes occurred on an ancestral β-thalassemia chromosome background.

The emerging picture from the study of the different nondeletion HPFH and $\delta\beta$-thalassemia syndromes favors a model where promoter up-mutations in the fetal domain on chromosomes with a normal adult domain result in an HPFH phenotype with an almost balanced synthetic ratio of α/non-α-globin chains. On the other hand, when promoter or other regulatory up-mutations occur in the fetal domain of chromosomes that carry down-mutations in the adult domain, the resulting phenotype is that of $\delta\beta$-thalassemia with imbalanced synthetic ratios of α/non-α chains. However, it is still not clear why the expression of the structurally normal β-globin gene in the nondeletion HPFH syndromes is decreased. It has been suggested that the mere presence of the fetal domain in an active transcriptional configuration may be enough to place the adult domain in a less active configuration.[27] The experimental tools for testing this hypothesis in vivo may soon become available. It is hoped that further studies of these nondeletion syndromes may continue to unravel more of the mysteries of globin gene regulation in mammalian cells.

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Further Characterization of Erythrocyte p80 and the Membrane Protein Defect of *In(Lu)* Lu(a − b −) Erythrocytes

By Marilyn J. Telen, Ian Rogers, and Michelle Letarte

We have previously shown that the *In(Lu)* gene down-regulates expression of an erythrocyte protein antigen identified by murine monoclonal antibody (MoAb) A3D8. In the present study we have examined *In(Lu)* Lu(a − b −) erythrocytes for expression of additional epitopes on the erythrocyte 80 kilodalton protein (p80) bearing the A3D8 antigen. Using a total of seven additional MoAbs that recognize three epitopes on erythrocyte p80, we have shown that *In(Lu)* Lu(a − b −) erythrocytes exhibit down-regulation of expression of all three epitopes. *In(Lu)* erythrocytes also showed a reduction in their reactivity to rabbit antibodies produced against purified p80 from either erythrocytes or lymphocytes. Furthermore the reactivity of the MoAbs was not altered by treatment of the cells with neuraminidase but was substantially reduced by treatment of cells with trypsin or chymotrypsin. The polyclonal anti-p80 sera were shown to react with a fragment of 50,000 daltons, still associated with erythrocyte ghosts, following treatment of the cells with trypsin or chymotrypsin. Treatment of erythrocytes with the thiol-reactive reagent AET decreased their reactivity with the MoAbs but had a variable effect on their reactivity with polyclonal antibodies. Erythrocyte p80 is a glycoprotein with N-linked oligosaccharides, as demonstrated by its binding to concanavalin A (Con A) and Len culinaris lectins. Following Endoglycosidase F treatment, erythrocyte p80 underwent a reduction in apparent mol wt of 11,000. The presence of a reduced amount of an intact p80 glycoprotein, seen by a decrease in reactivity with MoAbs directed at three distinct epitopes and with two different polyclonal antibodies, suggests that the *In(Lu)* gene interferes with expression by erythrocytes of the entire p80 glycoprotein.

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THE *IN(LU)* gene is an autosomal dominant gene that down-regulates but does not abolish expression of independently segregating genes encoding for the Lutheran family of erythrocyte antigens.[1,2] Once thought to be quite rare, this gene has now been shown to occur in some populations in as many as one in 3,000 donors.[3] The gene product and mode of action of the *In(Lu)* gene are not known. However, it has been recognized that the *In(Lu)* gene down-regulates expression of Lutheran antigens—which are sensitive to degradation by proteases[4] and by 2-aminoethylisothiouronium bromide[5]—and of the polysaccharide antigens P_1 and i.[6] We have also shown previously that the effect of the *In(Lu)* gene is not limited to erythrocytes.[7] Donors with the *In(Lu)* erythrocyte phenotype exhibit reduced expression of the A3D8-p80 antigen by erythrocytes as well as by a subset of leukocytes; these donors also have reduced amounts of antibody A3D8-reactive antigen in their serum.[7] The fact that the P_1 and i antigens are also down-regulated by the *In(Lu)* gene has prompted some investigators to hypothesize that the *In(Lu)* gene acts via a glycosyltransferase or blocks the activity of a glycosyltransferase.[8]

In this study we have utilized several antibodies to distinct epitopes of the p80 glycoprotein to demonstrate that the expression of all known epitopes was down-regulated by the *In(Lu)* gene.[7,9-12]

MATERIALS AND METHODS

Production of antibodies. The A3D8, A1G3, 50E6, 50C7, 50B4, 44D10, 50D7, and F10.44.2 antibodies are the products of previously described murine hybridoma cell lines.[7,9-12] Antibodies A3D8 and A1G3 have been shown to bind to nearby but nonidentical epitopes of the same protein of lymphocytes as well as of erythrocytes.[13,14] All other antibodies have been shown to bind to the same protein of lymphocytes.[14] In addition, competitive binding studies have shown that antibodies A3D8, F10.44.2, 50E6, and 50C7 bind to identical or indistinguishable epitopes[14]; antibodies 50B4, 44D10, and 50D7 bind to a second unrelated epitope on this protein.[14] Antibody A1G3 binds to a third epitope close to but not identical with that identified by A3D8 antibody.[13,14] All antibodies are of IgG1 or IgG2 heavy chain isotype.[7,9-12] Antibodies A3D8, A1G3, and F10.44.2 were used as ascites fluid diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). Antibodies 50E6, 50C7, 50B4, 50D7, and 44D10 were used as tissue culture supernates diluted in PBS-BSA. All binding studies were done under conditions of saturating amounts of anti-p80 antibodies.

Polyclonal antiserum to erythrocyte p80 was produced by injection of rabbits with p80 isolated from erythrocyte membrane lysates by affinity to A3D8-IgG-Sepharose. On day zero, 100 μg of antigen in complete Freund's adjuvant was injected subcutaneously (SC) at three sites; on day 19 this procedure was repeated, except that Freund's incomplete adjuvant was used. Two weeks after the second injection, serum was tested for binding to erythrocytes and was found to be reactive at dilutions up to 1:1,600. All data reported were obtained with this bleed diluted 1:25 in PBS-BSA; a pre-immunization rabbit serum was used as a control in all experiments.

Rabbit antiserum to lymphocyte p80 was produced by SC injection of purified protein obtained from a detergent extract of prolymphocytic leukemia cells by 50B4-IgG-Sepharose chromatography. For the first two immunizations (day 0 and day 21), 500 μg of antigen were given SC in complete Freund's adjuvant. The third immunization, on day 56, consisted of 300 μg reduced and alkylated antigen in complete Freund's adjuvant. The animal thereafter received booster inoculations consisting of 100 μg reduced and

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alkylated antigen eluted from sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and mixed with incomplete Freund's adjuvant. Experiments were performed with serum obtained on day 64 (bleed 3) and after the seventh injection (bleed 6). Antibody titer was measured by radioimmunoassay (RIA) using intact Lu(b+) erythrocytes. Both bleed 3 and bleed 6 saturated all binding sites at dilutions up to 1:200. Bleed 6 was reactive with reduced protein in Western blot, whereas bleed 3 was not. Binding assays were performed with saturating dilutions of antiserum (1:100) and preimmunization serum as a control.

Antibody binding assays. Erythrocyte cell surface reactivity with monoclonal antibodies (MoAbs) was measured by RIA or immunofluorescence as previously described.[7,15] Briefly, erythrocyte suspensions (5% vol/vol) were incubated 30 minutes at 4°C with dilutions of MoAbs or nonreactive control immunoglobulin (P3 × 63/Ag8 ascites or 50D6 tissue culture supernate, which contains MoAb to the HLA class II molecule).[16] Erythrocytes were then washed twice in 10 vol of PBS-BSA. For RIA, cells were resuspended to 150 μL and incubated with ^{125}I-labeled affinity purified F(ab')$_2$ antimouse Ig antibodies (Amersham Corp, Arlington Heights, IL) or ^{125}I-labeled staphylococcal protein A. Triplicate 50 μL aliquots of cell suspensions were then spun through phthalate oils (1.5 parts n-butyl phthalate [Fisher Scientific, Fair Lawn, NJ] to one part bis[2-ethylhexyl] phthalate [Eastman Kodak, Rochester, NY]). The centrifuged tubes were then clamped and cut, and the tips containing the cell pellets were counted in a gamma scintillation counter. RIA data were expressed as "Δ" cpm, as determined by the equation:

"Δ" cpm = mean cpm experimental − mean cpm control.

For immunofluorescence studies, cells were washed free of excess first antibody and then incubated with fluorescein-conjugated sheep antimouse IgG heavy and light chains (TAGO, Burlingame, CA, F/P > 6.0). After additional washes cells were resuspended in PBS-BSA and analyzed on a modified Ortho Cytofluorograf.

For analysis of the effects of proteolytic enzymes on the cell surface expression of p80 antigens, erythrocytes were treated with 2 mg/mL TPCK-trypsin (Worthington, Freehold, NJ) or chymotrypsin (Type II, Sigma Chemical Co, St. Louis) for 30 minutes at 37°C, washed three times with ice-cold PBS-BSA + 500 μg/mL soybean trypsin inhibitor, and then washed three times in PBS. Cells treated with ficin (Dr Madris Laboratories, South Hackensack, NJ) were incubated with 1 mg/mL enzyme for 30 minutes at 37°C and washed exhaustively with PBS.

Erythrocytes were treated with 12.5 U neuraminidase (from Vibrio cholerae, Calbiochem, La Jolla, CA) for 45 minutes at 37°C while suspended in Dulbecco's modified Eagle's Medium (DMEM, containing Ca^{++} and Mg^{++}) and then washed in PBS. Murine MoAb E5, which recognizes a sialic acid-dependent epitope on sialoglycoprotein β,[17] was used to assess the extent of neuraminidase digestion in these experiments.

To determine whether various p80 epitopes recognized by monoclonal and polyclonal antisera required intact disulfide bonds, erythrocytes were exposed to the thiol-reactive reagent 2-aminoethylisothiouronium bromide (AET) as previously described.[5]

After enzyme or AET treatment, cells were resuspended to 5% (vol/vol), and their level of reactivity with a series of antibodies was measured by RIA as above. Data given (Table 1) are either from a representative experiment (when three or more were performed) or represent the mean of two experiments. Aliquots of cells were also used to prepare membrane ghosts for immunoblotting experiments (see below).

Rare *In(Lu)* Lu(a−b−) erythrocytes were obtained from an inventory of frozen rare erythrocytes of the Duke Medical Center Immunohematology reference laboratory, from Dr M.N. Crawford[1] (Pearson Cummin Memorial Laboratory), and from Serologicals of Columbia, SC and Atlanta through the cooperation of Mary Ellen Rush and Nancy Fincher respectively. In all assays cells that were either frozen and thawed or cells that had undergone similar storage were used as Lu(b+) controls to eliminate any possible effects of age or storage on the expression of p80 antigens. At least three Lu(b+) controls were used in each experiment that examined *In(Lu)* erythrocyte antigens.

Immunochemical analysis of erythrocyte membrane proteins. Erythrocytes were washed free of serum and processed with or without enzyme treatment as described above. Erythrocytes were then made into hemoglobin-free ghosts by the method of Dodge et al[18] and solubilized in 100 mmol/L Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 15% glycerol. Samples containing 50 μg protein were then electrophoresed through 6% to 12% linear gradient SDS-polyacrylamide slab gels (PAGE) and electrophoretically transferred to nitrocellulose paper as previously described.[5] After incubation with 3% BSA in 150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4 to block nonspecific binding sites, the nitrocellulose was incubated with

Table 1. Effects of Enzymes and AET on Erythrocyte p80 Antibody Reactivity

	% of Normal Antibody Binding After Pretreatment of Erythrocytes with:				
Antibody	Chymotrypsin 2 mg/mL	Trypsin 2 mg/mL	Ficin 1 mg/mL	Neuraminidase	2-Aminoethylisothiouronium bromide
Monoclonal antibodies					
Epitope 1:					
A3D8	12	31	0	96	7
50E6	12	14		ND	0
Epitope 2:					
A1G3	9*	0†	1	118	1
Epitope 3:					
50B4	20	33	1	104	8
50D7	19	19	1	ND	4
Rabbit anti-erythrocyte p80	79	61	78	ND	159
Rabbit antilymphocyte p80					
Bleed 3	28	37	3	ND	35
Bleed 6	91	108	3	ND	93

Abbreviations: ND, not done.

*This assay was performed with 1 mg/mL chymotrypsin.

†Assayed by agglutination.

subsaturating dilutions of antibody. The reactive antigen-antibody complex was then localized by binding of rabbit antimouse Ig and murine monoclonal peroxidase-antiperoxidase complexes[19] or peroxidase-conjugated goat antirabbit Ig (Sigma), followed by color development with diaminobenzidine as substrate.

Purification of erythrocyte p80. A3D8 antibody was precipitated from ascites fluid by admixture with an equal volume of saturated ammonium sulfate, pH 7.4, and conjugated to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) (5 mg IgG per mL swollen gel). Ghosts were prepared from erythrocytes (washed free of serum), solubilized in an equal volume of 300 mmol/L NaCl, 10 mmol/L Na_2HPO_4, pH 7.4, 1% Nonidet P-40 (NP-40, vol/vol), 0.01% Tween 80 (wt/vol), 2 mmol/L PMSF, and centrifuged at 27,000 g for 20 minutes to remove insoluble material. A column of A3D8-Sepharose was equilibrated with a similar buffer containing 0.5% NP-40, and the soluble ghost extract was recycled through the column overnight at 4°C. The column was then washed with >5 column volumes equilibration buffer, followed by >5 column volumes 50 mmol/L Tris, pH 7.4. Elution of bound protein was accomplished with 2.5 mol/L $MgCl_2$, 50 mmol/L Tris, pH 7.4; the column was regenerated by washing with 0.1 mol/L Tris, 0.5 mol/L NaCl, pH 8.5, then 0.1 mol/L sodium acetate, 0.5 mol/L NaCl, pH 4.5, and finally 150 mmol/L NaCl, 10 mmol/L Tris, 0.1% NaN_3, pH 7.4. Eluted material was dialyzed sequentially against 50 mmol/L, 10 mmol/L, and 5 mmol/L Tris, pH 7.4 and lyophilized. Protein was then resolubilized in one-tenth volume of deionized water with 0.1% NP-40.

Lectin affinity chromatography. To assess the ability of erythrocyte p80 to bind to lentil (Lens culinaris) lectin, packed erythrocyte ghosts were mixed with an equal volume of 0.2 mol/L NaCl, 0.01 mol/L Tris-HCl, pH 7.4, 7 mmol/L $CaCl_2$ 7 mmol/L $MgCl_2$, 7 mmol/L $MnCl_2$, 0.5% NP-40 (vol/vol), 0.01% Tween 80 (wt/vol), 2 mmol/L PMSF. Lentil lectin-Sepharose 4B (Pharmacia) was equilibrated with a similar buffer containing 0.1% NP-40, and the membrane protein sample was applied to the column at room temperature. After washing the column with 5 volumes of equilibration buffer, bound proteins were eluted with equilibration buffer containing 0.1 mol/L α-methylmannoside. Eluates were concentrated by centrifugation in microconcentrators (Centricon-10, Amicon Corp, Danvers, MA).

Antigen eluted from A3D8-Sepharose was further purified by concanavalin A (Con A)-Sepharose chromatography. One hundred micrograms of protein was iodinated by incubation with 10 μg IodoGen (Pierce Chemical Co, Rockford, IL) and 1 mCi ^{125}I. The protein was then dialyzed against 0.1 mol/L Na_2HPO_4, 50 mmol/L EDTA, pH 7.0, to remove unbound ^{125}I. Radiolabeled protein was then applied to a Con A-Sepharose column (Pharmacia) equilibrated with a buffer containing 150 mmol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, pH 8.0, 0.1% Triton X-100. The column was washed in equilibration buffer until no cpm could be detected. Bound material was then eluted with a similar buffer containing 0.3 mol/L α-methylmannoside. Unbound and eluted materials were concentrated by dehydration with Aquacide (Calbiochem) and analyzed by SDS-PAGE followed by autoradiography.

Quantitation of p80 N-linked oligosaccharide. To obtain an estimate of the size of the N-linked oligosaccharides of p80 glycoprotein, Endoglycosidase F digestion was performed. Thirty-microgram aliquots of p80 purified by A3D8-Sepharose chromatography were solubilized in 0.1 mol/L sodium phosphate, 0.05 mol/L EDTA, 0.1% SDS, 1% NP-40, pH 6.1. Five units Endoglycosidase F were added to one aliquot of protein, and the enzyme-treated and control samples were incubated for two hours at 37°C. (It had previously been determined that 5 U enzyme was sufficient for a similar amount of glycophorin.) Aliquots were then mixed with SDS-PAGE sample buffer, electrophoresed, and analyzed by immunoblotting with A3D8 MoAb. For experiments performed under reducing conditions, radiolabeled p80 (approximately 13 μg per aliquot) was placed in the same buffer as above, except that 1% (vol/vol) 2-mercaptoethanol was added. Two units Endoglycosidase F were then added to one aliquot (keeping the enzyme:substrate ratio similar to that used in nonreducing conditions), and both aliquots were incubated for two hours at 37°C. Aliquots were then mixed with SDS-PAGE sample buffer, electrophoresed under reducing conditions, and the gels dried and exposed to x-ray film.

RESULTS

Characterization of epitopes recognized by antibodies to p80. We compared the effects of trypsin, chymotrypsin, and ficin treatment of intact erythrocytes on binding of A3D8, A1G3, 50E6, 50B4, and 50D7 MoAbs and on binding of polyclonal anti-p80 sera (Table 1) to determine the spatial relationships among the epitopes recognized by MoAbs and to assess the range of specificities recognized by the polyclonal antisera. Reactivity with all MoAbs was inhibited by treatment of intact erythrocytes with these proteases, indicating that there were cleavage sites for trypsin, chymotrypsin, and ficin in the extracellular domain of the protein and that cleavage at these sites destroyed the three epitopes recognized by MoAbs. The polyclonal antibodies, on the other hand, could still bind to erythrocytes after trypsin or chymotrypsin treatment, although only one polyclonal antiserum bound well after ficin treatment of red cells.

The epitopes recognized by antibodies 50B4, A3D8, and A1G3 were not sensitive to degradation by neuraminidase (Table 1). Binding of all MoAbs tested was markedly reduced by pretreatment of erythrocytes with AET, while this reagent had a more variable effect on reactivity of cells with polyclonal antisera (Table 1). The binding of rabbit anti-erythrocyte p80 to AET-treated erythrocytes was enhanced, while the binding of antilymphocyte p80 serum bleed No. 3 was decreased to 35% of normal. However, binding of antilymphocyte p80 serum bleed No. 6, obtained after several injections with dissociated, reduced and alkylated p80 glycoprotein, was decreased only 7% by AET treatment of erythrocytes. These data suggest that not all immunogenic epitopes of p80 are destroyed by AET treatment.

Binding of anti-p80 antibodies to In(Lu) *Lu(a−b−) erythrocytes.* Each of the eight MoAbs to p80 were tested quantitatively for their ability to bind to erythrocytes of Lutheran (b+) and *In(Lu)* Lu(a−b−) phenotypes by RIA, immunofluorescence-activated flow cytometry, or both, and results were compared to those obtained with multiple samples of Lu(b+) cells analyzed at the same time (Table 2). Antibodies 50E6 and 50C7, which bind to an epitope indistinguishable from that identified by antibody A3D8, were tested by RIA with nine and three *In(Lu)* samples respectively. *In(Lu)* erythrocytes exhibited a mean of 26% of normal 50E6 antibody binding, with a range of 9% to 38%. Antibody 50C7 also showed a mean of 26% of normal binding to *In(Lu)* erythrocytes (range 12% to 34%) by RIA. Antibody F10.44.2 (which also binds to an epitope indistin-

Table 2. Expression of p80 Antigens by *In(Lu)* Lu(a − b −) Erythrocytes

Antibody	Number of *In(Lu)* Lu(a−b−) Donors Tested	Mean % of Control Antibody Reactivity* Demonstrated by *In(Lu)* Lu(a−b−) RBC (Range %)
Epitope 1 ("A3D8-like")		
50E6	9	28 (25-38)
50C7	3	26 (12-34)
F10.44.2	3	25 (22-29)
Epitope 2		
A1G3	6	27 (23-31)
Epitope 3		
50B4	9	35 (13-44)
44D10	3	45 (29-58)
50D7	3	39 (29-45)
Rabbit anti-erythrocyte p80	2	10 (9-11)
Rabbit antilymphocyte p80		
Bleed 3	2	37 (22-45)

*As determined by antibody binding to erythrocytes from at least three Lu(b+) donors.

guishable from that identified by A3D8 antibody[14]) was tested for binding to Lu(b+) and *In(Lu)* erythrocytes by immunofluorescence-activated flow cytometry. The mean fluorescence channel (MFC) of *In(Lu)* erythrocytes stained with antibody F10.44.2 and fluorescein-conjugated anti-mouse antibody was 88 (on a linear scale), compared to an MFC of 357 for Lu(b+) cells, again showing that *In(Lu)* cells bound a mean of 25% (range 22% to 29%) as much anti-p80 antibody as normal cells, as measured by indirect immunofluorescence.

Antibodies 50B4, 44D10, and 50D7, which bind to an epitope on the p80 molecule unrelated to that identified by antibodies A3D8 or A1G3, were tested by RIA against Lu(b+) and *In(Lu)* Lu(a−b−) erythrocytes. *In(Lu)* erythrocytes bound 35% as much 50B4 antibody as did Lu(b+) cells and bound 45% and 39% as much 44D10 and 50D7 antibodies, respectively, as did normal cells (Table 2).

The polyclonal rabbit anti-erythrocyte p80 and rabbit antilymphocyte p80 sera were tested against samples of *In(Lu)* erythrocytes from the original *In(Lu)* proposita[1] and from her Lu(a−b−) nephew. Compared to normal Lu(b+) and Lu(a+b+) controls, the *In(Lu)* cells bound 10% as much polyclonal anti-erythrocyte p80 and 37% as much polyclonal antilymphocyte p80 (bleed 3).

Biochemical characterization of normal and In(Lu) *p80.* The erythrocyte p80 protein bound to lentil lectin, as observed for the lymphocyte protein.[10] Erythrocyte ghost proteins eluted from a lentil lectin-Sepharose affinity column contained sialoglycoproteins α and β, as well as erythrocyte p80, while sialoglycoprotein δ, as expected, was not bound to and eluted from lentil lectin (data not shown).

Fractionation of the NP-40/Tween 80 solubilized erythrocyte proteins by A3D8-Sepharose chromatography achieved isolation of p80 free from detectable contamination with glycophorins, band 3, and cytoskeletal components. As shown in Fig 1, eluted material analyzed by immunoblotting showed strong reactivity with A3D8 antibody but not with antibodies to sialoglycoproteins α, β, and δ. When an aliquot of material eluted from the A3D8-Sepharose column was radiolabeled and analyzed by SDS-PAGE under nonreducing conditions, a major band of mol wt = 80,000 was observed; a contaminating band of mol wt = 54,000 was also seen. Immunoblots of eluate stained with rabbit anti-p80 failed to demonstrate reactivity with the contaminating protein, suggesting that it was not likely a degradation product of p80. Purification of p80 away from the contaminating protein was achieved by fractionation on Con A-Sepharose (Fig 2); p80 was bound to Con A, while the contaminating protein remained in the unbound fraction.

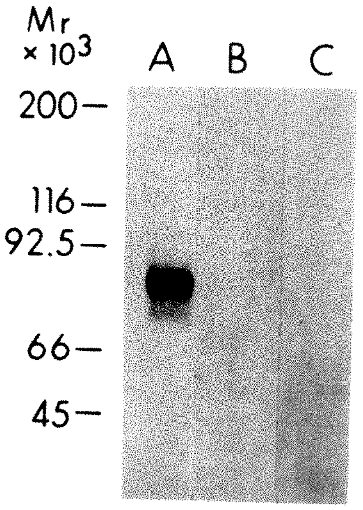

Fig 1. Immunoblot analysis of A3D8-affinity purified erythrocyte membrane protein. Erythrocyte membrane p80 was purified by A3D8-Sepharose chromatography as described in "Methods." Approximately 20 μg protein was then applied to each lane, analyzed by SDS-PAGE under nonreducing conditions, and transferred to nitrocellulose paper. Individual strips were then immunostained as described in "Methods" with MoAbs A3D8 (anti-p80, lane A), E3 (antisialoglycoproteins α and δ, lane B), and E5 (antisialoglycoprotein β, lane C). A3D8 reacted with a single protein band, while antibodies E3 and E5 failed to stain any protein.

Treatment of A3D8-purified antigen from a Lu(a−b+) donor with Endoglycosidase F showed that, if not treated with 2-mercaptoethanol, erythrocyte p80 was extremely resistant to the action of Endoglycosidase F. Under conditions that produced complete removal of the N-linked carbohydrate moiety of sialoglycoprotein α (glycophorin A), only a minor portion of the p80 was affected (Fig 3). However, removal of N-linked carbohydrate could be achieved in the presence of 2-mercaptoethanol during the enzymatic digestion. A decrease in apparent mol wt of radiolabeled erythrocyte p80 of approximately 11,000 daltons was observed following removal of N-linked oligosaccharides (Fig 3).

By using the polyclonal rabbit anti-p80 sera, we were able to demonstrate the cell-bound tryptic and chymotryptic fragments of p80 obtained following treatment of intact cells (Fig 4). Cells treated with 2 mg/mL trypsin showed a decrease in p80 but expressed a 50 kd polypeptide reactive with rabbit anti-p80. Chymotrypsin treatment produced a similar polypeptide fragment reactive with anti-p80. These fragments were unreactive with any of the MoAbs, consistent with cell binding data suggesting that all the epitopes recognized by the MoAbs resided either on the portion of the

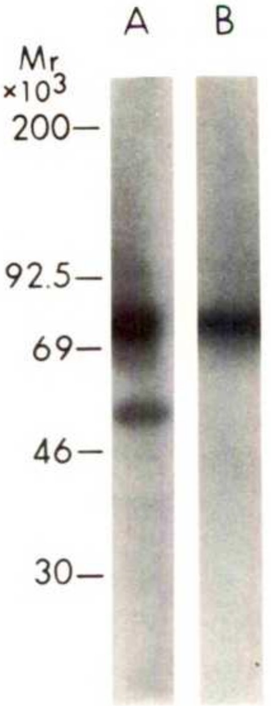

Fig 2. Analysis by radiolabeling and SDS-PAGE of the A3D8 antigen and further purification using Con A. Antigen eluted from A3D8-Sepharose was radiolabeled with ^{125}I and an aliquot analyzed by SDS-PAGE. Lane A shows the radiolabeled protein under nonreducing conditions, consisting of a major band at mol wt 80,000 and a minor band at mol wt 54,000. A portion of radiolabeled protein was then passed through a Con A-Sepharose column, as described in "Methods." SDS-PAGE analysis of the material eluted with α-methylmannoside, as shown in lane B, demonstrated a single band with mol wt = 80,000.

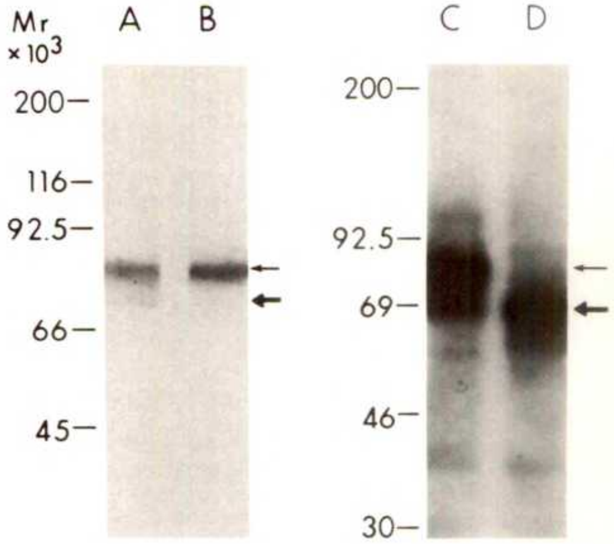

Fig 3. Endoglycosidase F digestion of erythrocyte p80. The presence of N-linked oligosaccharide on erythrocyte p80 was assessed by exposure to Endoglycosidase F. Ten micrograms of purified A3D8 antigen was incubated (in the absence of 2-mercaptoethanol) for two hours with either buffer alone (lane B) or buffer with 5 U Endoglycosidase F (lane A). Material was then analyzed by SDS-PAGE under nonreducing conditions and immunoblotted with A3D8 antibody. Only a small amount of material showed a decrease in apparent mol wt (bold arrow), while most of the protein remained intact (light arrow). However, when radiolabeled p80 protein was treated with 2-mercaptoethanol prior to Endo F digestion and analyzed by SDS-PAGE under reducing conditions, all native protein (lane C, light arrow) exhibited a reduction in apparent mol wt of about 11,000 (lane D, bold arrow).

molecule cleaved from the intact cell by these enzymes or on a portion whose secondary structure is dependent on the presence of the cleaved fragment.

Rabbit antilymphocyte p80 was used to immunoblot *In(Lu)* Lu(a−b−) erythrocyte membranes to determine whether *In(Lu)* cells contained an abnormal p80 protein not detectable with MoAbs (Fig 5). In immunoblots of normal and *In(Lu)* membrane proteins, both bleed 3 and bleed 6 rabbit antilymphocyte p80 reacted with a single glycoprotein of mol wt = 80,000 and failed to detect additional anomalous protein bands. As previously observed with MoAbs, reduced amounts of p80 were present in *In(Lu)* membrane protein extracts compared to extracts from Lu(b+) erythrocytes.

DISCUSSION

This study has shown that the *In(Lu)* gene down-regulates the expression of erythrocyte p80, as detected by MoAbs recognizing three distinct epitopes and by polyclonal antisera. *In(Lu)* Lu(a−b−) erythrocytes bound reduced amounts (10% to 45%) of each anti-p80 antibody tested when compared to Lu(b+) or recessive Lu(a−b−) cells. The fact that distinct antibodies to p80 showed somewhat different reductions in binding to *In(Lu)* erythrocytes could be due to variations in antibody affinity or actual variations in the presence of these epitopes on *In(Lu)* cells. However, because all MoAbs appear to recognize epitopes dependent on maintenance of intrachain disulfide bonds (as indicated by reduced binding to erythrocytes treated with AET), it is most likely that they bind protein determinants dependent on the secondary structure of p80. Therefore the binding data are most consistent with the hypothesis that expression of the entire p80 molecule is decreased on *In(Lu)* Lu(a−b−) erythrocytes. This hypothesis is further supported by our data that the polyclonal antisera to p80 failed to detect immunoreactive protein other than p80 in immunoblots of *In(Lu)* erythrocyte membrane proteins.

The ability of lentil lectin and Con A to bind erythrocyte p80 showed that it is a glycoprotein with N-linked oligosaccharides, as demonstrated previously for lymphocyte p80.[10] This observation is of interest because the biochemical nature of Lutheran antigens is as yet unknown and because the *In(Lu)* gene has also been reported to down-regulate expression of the P_1 and i polysaccharide antigens. It thus remains to be shown whether individual Lutheran antigens might arise from variations in amino acid sequence of a protein, variations in glycosylation, or a combination of these. Although preliminary evidence shows that Lutheran antisera immunoprecipitate and immunoblot a protein of nearly the same mol wt as does MoAb A3D8 (Telen, unpublished observations, and Parsons et al,[20] respectively), no definitive evidence is yet available regarding whether the erythrocyte protein identified by A3D8 antibody is identical

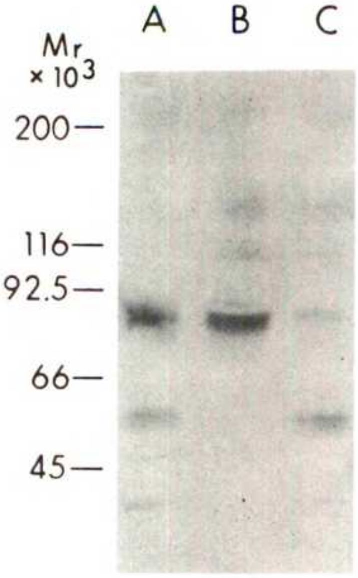

Fig 4. Cleavage products of erythrocyte p80 following protease treatment of intact cells. Erythrocytes were treated with trypsin, chymotrypsin, or buffer only, and ghosts were prepared as described in "Methods." Ghost proteins were then solubilized, analyzed by SDS-PAGE, and immunoblotted using rabbit anti-p80. Control erythrocytes demonstrated intact p80 (lane B). Cells treated with trypsin (lane A) showed residual p80 and a major fragment of mol wt 50,000, while cells treated with chymotrypsin (lane C) contained primarily a single immunoreactive peptide of mol wt 50,000.

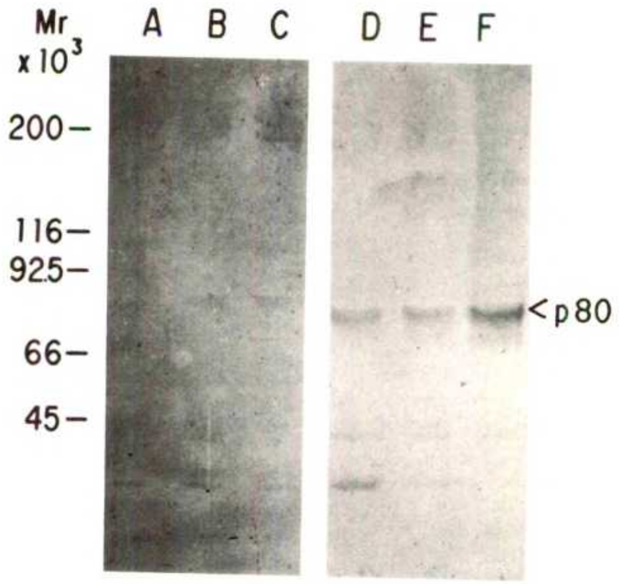

Fig 5. Immunoreactive p80 protein of Lu(b+) and *In(Lu)* erythrocytes. Ghost proteins from Lu(b+) erythrocytes (lanes C and F) and two donors' *In(Lu)* erythrocytes (lanes A and D, lanes B and E) were analyzed by SDS-PAGE and immunoblotting for presence of protein reactive with rabbit antilymphocyte p80 bleed 3 as described in "Methods." Each lane was overloaded with protein (80 μg/land) to increase sensitivity for small amounts of anomalous protein. Lanes A, B, and C were stained with preimmunization serum, and lanes D, E, and F were stained with rabbit antiserum to p80. *In(Lu)* erythrocyte ghosts contained less p80 than normal erythrocyte ghosts but did not demonstrate immunoreactive protein bands at anomalous positions on the gel. Similar results were obtained with rabbit antilymphocyte p80 bleed 6 (data not shown).

to that which binds Lutheran antisera. Ficin treatment of red cells abolished binding of p80 MoAbs but does not abrogate binding of at least some Lutheran antisera. However, these data are insufficient to determine whether these antigens reside on different proteins or at spatially distant sites on the same protein.

The fact that the *In(Lu)* gene down-regulates the expression of several epitopes on the p80 glycoprotein suggests that it is not likely to block the expression of a single oligosaccharide antigenic determinant unless the absence of such a determinant caused failure of insertion of the protein into the cell membrane. To date we have established that three distinct epitopes recognized by MoAbs were not affected by treatment of cells with neuraminidase. The *In(Lu)* gene seems to decrease the level of expression of erythrocyte p80 at the cell surface rather than lead to the production of an abnormal p80 protein. Multiple epitopes on the protein are down-regulated by the *In(Lu)* gene, while no anomalous protein is detectable in *In(Lu)* erythrocyte membrane protein. The observation that a single copy of the *In(Lu)* gene depresses expression of p80 antigens by greater than 50% suggests that the *In(Lu)* gene acts by interfering with transcription or with translation, processing, or insertion into the membrane of a normally encoded protein, whether this protein is encoded by the *in(Lu)* [normal] gene or another gene. Alternatively, the protein could be destabilized in the presence of the *In(Lu)* gene due to an abnormal interaction with another moiety resulting more directly from the action of the *In(Lu)* gene.

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Elimination of Clonogenic Stem Cells From Human Multiple Myeloma Cell Lines by a Plasma Cell-Reactive Monoclonal Antibody and Complement

By Alex W. Tong, Jennifer C. Lee, Joseph W. Fay, and Marvin J. Stone

The monoclonal antibody (MoAb) MM4 reacts with human multiple myeloma (MM) cell lines and bone marrow from patients with plasma cell dyscrasias but not with normal peripheral blood or bone marrow cells. Treatment with MM4 and rabbit complement (C′) was cytotoxic to the plasma cell-derived cell lines GM 1312, RPMI 8226, and ARH-77, as demonstrated by chromium release microcytotoxicity and trypan blue exclusion assays. The same treatment eliminated >99% of clonogenic myeloma stem cell colony formation of these cell lines, with less than 20% inhibition of normal human bone marrow pleuripotent progenitor colony formation in vitro. As an experimental model to explore the efficacy of MM4 + C′ in purging MM-involved bone marrow, normal marrow cells were mixed with RPMI 8226 or GM 1312 cells in the ratio of 90:10 or 50:50 (marrow:myeloma cells). Colony growth assays indicated that MM4 + C′ eliminated at least 2 logs of clonogenic myeloma stem cells in both 90:10 and 50:50 preparations, while sparing the majority of normal marrow progenitors (inhibition of CFU-C:10% to 13%; BFU-E:0%). The selectivity of MM4-mediated cytotoxicity may be useful for eliminating myeloma clonogenic stem cells from bone marrow of patients with multiple myeloma.

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MULTIPLE myeloma (MM) represents a malignant plasma cell dyscrasia (PCD) with a wide spectrum of clinical manifestations.[1-8] In 1986 it was estimated that approximately 10,000 new patients were diagnosed with this disorder in the United States. With the use of alkylating agent chemotherapy regimens, objective remission is possible for 30% to 60% of the myeloma patient population.[8] Despite improvement in quality and duration of remission, the median survival for MM patients is limited to 3 to 4 years from time of diagnosis and has not improved substantially during the past 20 years.[1-8] MM is in the advanced stages of its natural history at time of diagnosis,[1-8] and patients responding to therapy rarely have more than a 1- to 2-log reduction (90% to 99%) of their neoplastic mass.[1,8] A large residual tumor burden, or the emergence of a drug resistant subclone,[9] accounts for the high incidence of tumor relapse. Patients with MM usually die of progressive disease, infections due to immunosuppression commonly associated with MM, or renal failure.[5-10]

Adjuvant bone marrow (BM) transplantation can provide the option of extending the dose range of cytotoxic chemotherapeutic agents to improve patient chemoresponsiveness and to prolong survival.[11] This approach has proven effective for some patients with primary hematopoietic malignancies, including refractory non-Hodgkin's lymphomas[11,12] and acute and chronic granulocytic leukemias.[12,13] Marrow transplantation studies on MM patients have been limited.[14,15] In a clinical trial of five MM patients, transplantation of syngeneic marrow accompanying massive chemotherapy extended survival.[15] However, the likelihood of success using allogeneic marrow transplantation is limited by age and/or performance considerations of the MM patient population.[1-7] Whereas appropriate monoclonal antibodies (MoAbs) can serve as tumor-purging reagents for preparing autologous BM for transplantation in other hematologic malignancies,[16-20] this approach has not been extensively investigated in MM. The majority of plasma cell-reactive MoAbs described also react with granulocytes[21-23] or lymphocytes[22-26] or do not bind the majority of patient myeloma cells.[23-26]

In a previous report[27] we described a myeloma cell-reactive MoAb, MM4, generated in this laboratory. MM4 reacted with a 40-kd antigenic determinant that was detected on seven of seven human plasma cell-derived lines and on the majority of plasma cells in all of 18 BM biopsies from patients with various plasma cell dyscrasias (MM, Waldenström's macroglobulinemia, or asymptomatic plasma cell dyscrasia). By contrast, MM4 did not react with BM biopsies or peripheral blood mononuclear (PBM) cells from patients with lymphocytic or granulocytic leukemias. The MM4-reactive antigen also was not expressed on normal PBM cells, granulocytes, or erythrocytes.[27]

In this study we examined the complement-mediated cytotoxicity of MM4 with plasma cell-derived cell lines, normal peripheral blood, and bone marrow cells. In an experimental model of normal bone marrow mixed with cells from different human MM-derived cell lines, MM4 + C′ eliminated myeloma clonogenic stem cells while preserving most normal BM progenitor cells. These observations suggest that this plasma cell-reactive MoAb may be of value for selectively eliminating myeloma cells from MM marrow ex vivo.

MATERIALS AND METHODS

Human multiple myeloma-derived cell lines. The plasma cell line GM 1312 was obtained from the NIGMS Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, NJ. The multiple myeloma-derived RPMI 8226, plasma cell leuke-

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mia-derived ARH-77, and plasmacytoma-derived HS-Sultan lines were obtained from the American Type Culture Collection, Rockville, MD, as described previously.[27] The mouse myeloma cell line SP2/0-Ag14 was obtained from Dr Denis Burger, Portland, OR.

Patient blood and bone marrow specimens. Human PBM cells and BM samples from healthy controls or patients with hematologic malignancies were procured according to the protocol approved by the Institutional Review Board for Human Protection, Baylor University Medical Center (BUMC).

Patients with hematologic malignancies were diagnosed and classified independently by the clinical staff, Department of Oncology, Charles A. Sammons Cancer Center, BUMC. Sections of paraffin-embedded BM biopsies from these patients were obtained from the Department of Pathology, BUMC, after routine histopathologic evaluation of the specimens.

Preparation of BM + MM cell mixtures. Normal marrow mononuclear cells were derived from Hypaque Ficoll centrifugation (400 g, 20 minutes) of marrow aspirates and resuspended in RPMI 1640 at 10^6/mL.[27] For the preparation of BM + MM cell mixtures, these were mixed with appropriate volumes of either RPMI 8226 or GM 1312 cells (10^6/mL) to achieve normal marrow:myeloma cell ratios of 90:10 or 50:50.

Production of MoAbs. MM4 was generated by an alternate immunization protocol, as described previously.[27-29] Briefly, BALB/c mice were immunized alternately with 1×10^7 live cells from the RPMI 8226 and GM 1312 cell lines. Somatic hybridization of the sensitized splenocytes with SP2/0-Ag14 myeloma cells (1:1, splenocyte:myeloma cells) was performed three days after the last injection, utilizing 50% polyethylene glycol and 5% dimethyl sulfoxide according to the method of Tong et al[27-29] and Oi and Herzenberg.[30] Syngeneic thymocytes served as "filler cells" (10^6 per well) during propagation. Antibody reactivity of confluent hybridoma cultures was characterized by ELISA against MM cell lines and PBM cells from normal donors as previously described.[27-29] Selected hybrid clones were carried in vivo by injecting 2×10^7 cells intraperitoneally in pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemicals, Milwaukee)-primed BALB/c mice. MM4 from ascitic fluid was partially purified by precipitation with saturated ammonium sulfate solution and adjusted to a protein concentration of 10 mg/mL from an initial concentration of 15 to 20 mg/mL.

Reactivity of MM4 with MM cell lines and peripheral blood cells was determined by an ELISA assay.[27] Reactivity with Zenker formalin-fixed bone marrow biopsies was assessed by the immunoperoxidase assay, as described previously.[27-29]

Microcytotoxicity assay. Cytotoxicity assays were performed according to modifications of the method described by Steplewski et al[31] and others.[32,33] Target cells were labeled with sodium ^{51}chromate (New England Nuclear, Boston; 200 μCi per 2×10^6 cells, 37°C, two hours), washed twice with serum-free media, then incubated for another 30 minutes with RPMI 1640 and 10% fetal calf serum (FCS) to reduce nonspecific background radioactivity. Treatment of the washed cells (10^5/mL in RPMI 1640 + 10% FCS) with an equal volume of MM4 at the indicated final concentration was performed at 4°C (60 minutes) to minimize antigenic modulation.[34] The cells were washed once, resuspended to 10^5 cells/mL, and dispensed into a **96-well microtiter plate (100 μL/well). Complement from 3- 4-week old rabbits (Lot No. 1007; Pel-Freez, Brown Deer, WI) was** preadsorbed with human AB erythrocytes (4°C, 60 minutes) to minimize background toxicity. Incubation with complement was carried out at room temperature (100 μL per well, 23°C, 30 minutes), as defined by pilot experiments that indicated that a change of incubation temperature from 37°C reduced nonspecific toxicity without appreciably affecting MM4-specific lysis. The microtiter plate was centrifuged briefly to pellet the cells (100 g, five minutes), and half the supernatant fluid from each well was withdrawn to determine chromium release by the lysed target cells. The percent-specific lysis was determined by comparing chromium release in treated samples (CPM_{test}) with maximal release (CPM_{max}: 0.5% NP40-treated target cells), after subtraction of background (CPM_{bkg}; cells untreated with MoAb or complement), by the formula:

$$(CPM_{test} - CPM_{bkg})/(CPM_{max} - CPM_{bkg}) \times 100\%.$$

Trypan blue exclusion. The proportion of viable MM cells after MM4 + C′ treatment was determined by trypan blue exclusion.[35] Treated samples were washed once with phosphate-buffered saline (PBS), then resuspended in 200 μL of 0.5% trypan blue in saline. Viability was established by the number of cells that excluded the dye, with at least 200 cells counted in a hemocytometer.

Myeloma clonogenic stem cell assay. The clonogenic stem cell assay for cells from PCD-derived cell lines was performed according to a modified technique of Hamburger and Salmon.[36] Incubation with MM4 (4°C, 60 minutes) was performed after pelleting the tumor cells in a sterile 15-mL centrifuge tube (American Scientific Products, Dallas). The MoAb-treated cells were washed twice in RPMI 1640, incubated with rabbit complement at room temperature, then distributed in triplicates onto a 6-well culture plate (Falcon) in 0.3% agar with Iscove's modified Dubecco's medium (IMDM) and 15% FCS. The cultures were propagated in a 37°C incubator, and the number of colonies (with >50 cells) were enumerated under low magnification after seven days. The percent of growth inhibition was determined by comparing MM4-treated cultures with those treated with the same concentration of an irrelevant MoAb (SCLC 1096) that reacts with human small-cell lung carcinoma, but not with human normal peripheral blood or myeloma cells.[27,28]

BM progenitor colony formation. As a means of monitoring normal bone marrow precursor function, CFU-C and BFU-E colonies were examined in untreated and MM4 + C′-treated bone marrow preparations.[37] One hundred microliters of bone marrow mononuclear cells (5×10^5/mL) were layered onto a culture plate gelatinized with 1 mL of methylcellulose culture media (0.8% methycellulose, 20% FCS, 0.3 mmol/L 2-mercaptoethanol), and incubated at 37°C in 100% humidity, 5% CO_2, with GCT-conditioned medium (100 μL/mL; GIBCO, Grand Island, NY) for stimulating granulocytic precursor colony formation. Erythropoietin (Connaught Laboratories, Willowdale, Ontario, Canada) at a final concentration of 2.5 IU/mL was added on day 4. CFU-Cs were identified by their lack of color and relatively diffuse pattern and BFU-Es by their red color and tightly packed colonies.[37]

For the study of cell preparations containing both normal bone marrow and MM cells, myeloma clonogenic stem cells were distinguished by their larger colony size, faster growth rate, and distinctive colony morphology. GM 1312 colonies were characterized by their tightly packed spherical conformation, whereas RPMI 8226 colonies were comprised of tightly aligned tumor cells. The immunologic phenotype of cells from myeloma colonies was verified by reactivity with the plasma cell-reactive MoAbs PCA-1 (Coulter Immunology, Hialeah, FL), OKT10 (Ortho Diagnostic Systems, Boston), and MM4. Cells from myeloma colonies were removed under an inverted microscope by a drawn Pasteur pipette at day 7, deposited on slides (5×10^5 per slide) by cytospin centrifugation (Shandon Southern, Sewickeley, PA), and treated with Saccomanno fixative (50% ethanol + 2% polyethylene glycol). Reactivity with the various plasma cell-reactive MoAbs was determined by the immunoperoxidase technique.[27-29]

Limiting dilution analysis. Limiting dilution analysis of clonogenic units was determined using a Spearman estimate.[17,38] A series of ten fivefold dilutions were prepared from MM4 + C′-treated or untreated normal bone marrow preparations containing 10%

myeloma cells from the cell lines RPMI 8226, GM 1312, or ARH-77 that initially contained 5×10^5 tumor cells/mL. Five replicates (100 μL) of each dilution were plated in 6-well flat-bottomed tissue culture plates (Falcon) onto methylcellulose under the culture conditions described above. The mean value for each treatment group was estimated by:

$$M = X_o + d/2 - d/n \sum_{i=0}^{k} r_i$$

where X_o = ln initial dose = ln 10; d = ln dilution factor = ln 5; n = number of replicates per dilution = 5; k = number of fivefold dilutions = 9; and r_i = the number of wells with observed growth at the ith dilution. The estimated number of clonogenic units per milliliter was calculated as $\Theta = \exp(-0.5772 - M)$, with variance approximated by d ln 2/n.

RESULTS

MM4-mediated cytotoxicity. Cytotoxicity of MM4 and C′ against cells from established plasma cell dyscrasia (PCD)-derived cell lines was determined by short-term chromium release assays. Exposure to the IgG1 MoAb MM4 at 0.015 to 1 mg per 10^5 cells followed by rabbit C′ at the pretitered final dilution of 1:16 was cytolytic to 25% to 95% of cells from the PCD cell lines tested (Fig 1). The LD50s of MM4 + C′-mediated cytotoxicity were 0.015 mg, 0.047 mg, and 0.045 mg for GM 1312, RPMI 8226, and ARH-77 cells respectively, with a dose-response pattern of the ARH-77 line almost identical to that of RPMI 8226 (Fig 1). Toxicity to normal PBM cells (20% ± 7.5%) was evident after incubation with MM4 at 1 mg. Subsequent determinations thus were conducted at the optimal MoAb concentration of 0.5 mg/10^5 cells.

Similar to the observations of Bast[17] and others,[16,18,19] two cycles of C′ incubation (1:16 final dilution) improved cytolysis of MM4-bound tumor cells to 94.5%, 72.3%, and 80.5% for GM 1312, RPMI 8226, and ARH-77 lines respectively (Table 1). There was no appreciable increase in background toxicity by C′ alone, with a mean cytolysis of 18.75% ± 5.25% with the three cell lines tested. Under the same experimental conditions, evaluations by trypan blue exclusion demonstrated that <1% of GM 1312 cells were viable (Table 1) after MM4 + C′ treatment, despite a lower level of chromium release (94.5%). The same treatment yielded less than 6% viable RPMI 8226 cells, according to trypan blue exclusion studies. Higher background toxicity and a corresponding increase in normal BM and PBM cell lysis were observed if complement treatments were extended to three or more cycles (data not shown) or if a higher concentration of complement was used (2 × 1:8 or 1:4, Table 1). Such variations also did not result in significant improvement of cell kill, as determined by trypan blue exclusion (Table 1). Re-exposure to MM4 after complement treatment did not enhance myeloma-specific cytolysis.

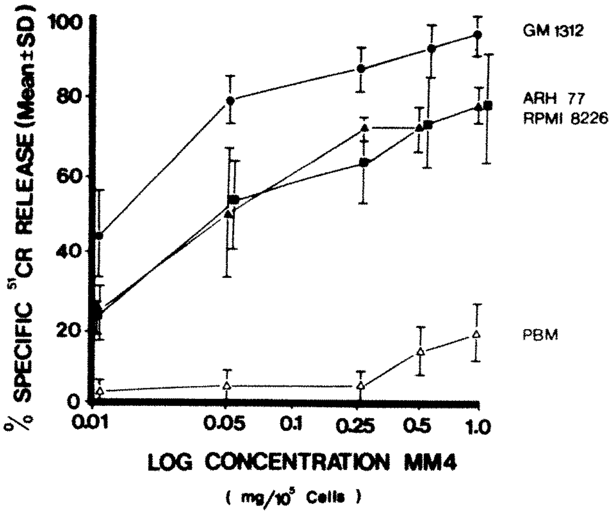

Fig 1. MM4 and complement-mediated cytolysis of MM-derived cell lines. Percent specific ^{51}chromium release by prelabeled MM cell lines GM 1312 (●), RPMI 8226 (▲), and ARH-77 (■), as well as peripheral blood mononuclear cells (PBM: △) was determined after incubation with the indicated concentration of MM4 and rabbit complement at 1:16 (see "Materials and Methods").

Depletion of clonogenic stem cells from MM cell lines. Tumor repopulation and/or metastatic growth have been postulated to be mediated by a self-renewing tumor stem cell subset. Chemoresponsiveness of clonogenic stem cell cultures of patients in vitro has been correlated with subsequent clinical response.[39-41] Previously, MM4 alone has been found to inhibit clonogenic colony formation of RPMI 8226 cells, with an LD50 of 0.01 to 0.1 mg/10^5 cells.[27] We carried out clonogenic stem cell assays to establish the effect of MM4 + C′ on clonogenic stem cells from PCD cell lines. MM4 + C′ completely abrogated myeloma clonogenic stem cell colony formation of GM 1312, RPMI 8226, and ARH-77 cell lines under optimized conditions (Table 1). The inhibition of normal marrow stem cell colony formation (CFU-C) under the same conditions was limited to 14.6% and 7.5%, after exposure to complement at 1:8 and 1:16 dilutions respectively.

Elimination of myeloma cells from bone marrow. As an experimental model to test the efficacy of MM4 + C′ in eliminating myeloma cells from involved patient marrow, cells from the RPMI 8226 or GM 1312 lines were mixed with normal bone marrow mononuclear cells in final proportions of 50:50 and 90:10 (normal marrow:myeloma cells). These ratios were designed to approximate the content of myeloma cells in BM of patients prior to treatment and in remission.

As shown in Table 2 with GM 1312 cells, the efficacy of MM4 + C′ in eliminating myeloma clonogenic stem cells from the tumor cell line was not affected by the presence of normal BM cells (Table 2). However, the overall capacity of this treatment in lysing tumor cells was diminished based on chromium release data. A mean cytolytic value of 83.8% and 59.5% of chromium-prelabeled GM 1312 cells was observed in preparations containing 90% or 10% of unlabeled BM cells respectively (Table 2).

The cytotoxic effect of MM4 + C′ on clonogenic stem cells of established MM cell lines and normal BM progenitors was examined under different incubating conditions (Figs 2 and 3). MM clonogenic stem cell colonies, enumerated at day 7, were distinguished from normal BM stem cell colonies by their characteristic colony morphology (Fig 2) and by their immunologic reactivity with plasma cell-

Table 1. Effect of MM4 + Complement Treatment on Multiple Myeloma Cell Lines and Normal Bone Marrow

Target	Cytolysis*		% Nonviable Cells†		% Inhibition Of Colony Formation‡	
	1:8	1:16§	1:8	1:16	1:8	1:16
MM cell lines						
GM 1312	96.1 ± 3.1	94.5 ± 4.6	97.1 ± 3.9	99.5 ± 1.0	100 ± 0	100 ± 0
RPMI 8226	80.8 ± 3.3	72.3 ± 2.5	93.5 ± 5.6	94.3 ± 8.5	100 ± 0	100 ± 0
ARH-77	88.0 ± 4.3	80.5 ± 2.2	NT‖	NT	100 ± 0	100 ± 0
Normal bone marrow	34.3 ± 8.7	20.1 ± 8.7	NT	NT	14.6 ± 9.9	7.5 ± 10.4

*Determined by % specific ^{51}chromium release microcytotoxicity assay after incubation with MM4 (0.5 mg/10^5 cells; 4°C, 60 minutes) and rabbit complement (23°C, 30 minutes × 2) at the indicated final dilution. Value represents mean ± SD (n = 5).

†Determined by trypan blue exclusion (see Materials and Methods).

‡Determined by stem cell colony-growth assay for treated myeloma cell lines and CFU-C colony formation for normal bone marrow. Myeloma stem cell colonies were scored on day 7, and CFU-C on day 10. Value represents Mean ± SD (n = 4).

§Final complement dilution.

‖NT, not tested.

reactive MoAbs PCA-1, OKT10, and MM4. For preparations containing 10% myeloma cells (Fig 3 A), MM4 + C′ (1:16) consistently reduced GM 1312 clonogenic stem cell colonies by at least 2 logs (99% to 100%, n = 4). Similarly, RPMI 8226 stem cell colonies were reduced by 97% to 100% (98.6% ± 1.4%, n = 3). The same treatment inhibited 13% of normal bone marrow CFU-C but did not affect BFU-E progenitor growth. Unlike incubation of MM or BM cells alone, increase of C′ concentration to 1:8 depleted MM clonogenic stem cell colonies from both MM cell lines but did not significantly increase toxicity to BM progenitor colony formation, as measured by CFU-C and BFU-E (Fig 3 A).

Similarly, in BM preparations containing 50% MM cells (Fig 3 B), MM4 + C′ at 1:16 completely eradicated colony formation by GM 1312 and eliminated 90% (90.2% ± 14.7%, n = 3) of RPMI 8226 clonogenic stem cells. Similar to our observations on 90:10 cell mixtures, incubation with C′ at 1:8 completely inhibited RPMI 8226 clonogenic stem cell colony formation without significantly increasing toxicity to normal BM CFU-C (% inhibition: 19.7% ± 11.1%, n = 4). A slight increase in toxicity to BFU-E (% inhibition: 9.7%, n = 2) was observed at this complement concentration.

To better establish efficacy of clonogenic stem cell elimination under optimized conditions, myeloma clonogenic units in marrow preparations containing 10% myeloma cells before or after MM4 + C′ treatment were determined by limiting dilution analysis (Table 3). Two or more log reductions of clonogenic stem cell units were observed (99.4%, 98.9%, and 99.97% reduction for RPMI 8226, GM 1312, and ARH-77 cells respectively). Similar evaluation with the plasmacytoma-derived cell line HS-Sultan also demonstrated a nearly 3-log depletion of clonogenic stem cells (Table 3).

DISCUSSION

The MoAb MM4 described in this study recognizes a 40-kd antigenic determinant that is commonly expressed on normal and neoplastic plasma cells.[27] The pattern of reactivity of MM4 differs from most other previously described plasma cell-reactive MoAbs,[21-26,42] including PCA-1 and PCA-2,[21,22] R1-3,[24] B5,[25] common acute lymphoblastic leukemia antigen (CALLA),[23] MB-1,[26] and OKT10.[22] Based on enzyme-linked immunosorbent assays, or immunoperoxidase and immunofluorescence evaluations under optimized conditions, MM4 does not react with normal BM cells or with peripheral blood normal T or B lymphocytes, granulocytes, or erythrocytes but binds to the majority of plasma cells in all plasma cell-derived cell lines and plasma cell dyscrasia BM biopsies tested.[21] In this study we examined whether this MoAb may be utilized as a means of selectivity eliminating myeloma cells in the presence of rabbit complement.

As a means of controlling for possible cell culture artifacts, the cytolytic properties of MM4 were examined with cells from three different plasma cell-derived cell lines. According

Table 2. Effect of MM4 + Complement Treatment on Cell Preparations Containing Normal Bone Marrow and Myeloma Cells

BM:GM 1312*	MM4 Added (mg)	GM 1312 Cells: % Colony Inhibition†	GM 1312 Cells: % Specific ^{51}Cr Release‡	% Inhibition of BM Stem Cells§: CFU-C	% Inhibition of BM Stem Cells§: BFU-E
90:10	0.5	99.5 ± 0.5	83.8 ± 9	13.5 ± 10	0
	0.25	95.1 ± 4	78.4 ± 11	10.2 ± 10	0
50:50	0.5	100 ± 0	59.5 ± 14	13.3 ± 13	0
	0.25	92.4 ± 4	51.5 ± 5	7.1 ± 7	0

*GM 1312 cells were mixed with normal bone marrow mononuclear cells in the indicated ratios.

†Determined by stem cell colony-growth assay on day 7 and differentiated from marrow colonies by their distinctive colony morphology and irregular growth patttern. Value represents mean ± SD (n = 4).

‡Determined by short-term microcytotoxicity assay (see Materials and Methods). Mean ± SD (n = 3).

§CFU-C colonies were scored on day 10 and identified by their lack of color and relatively diffuse pattern. BFU-E were recognized by their red color and tightly packed colonies on day 14. Value represents mean ± SD (n = 4).

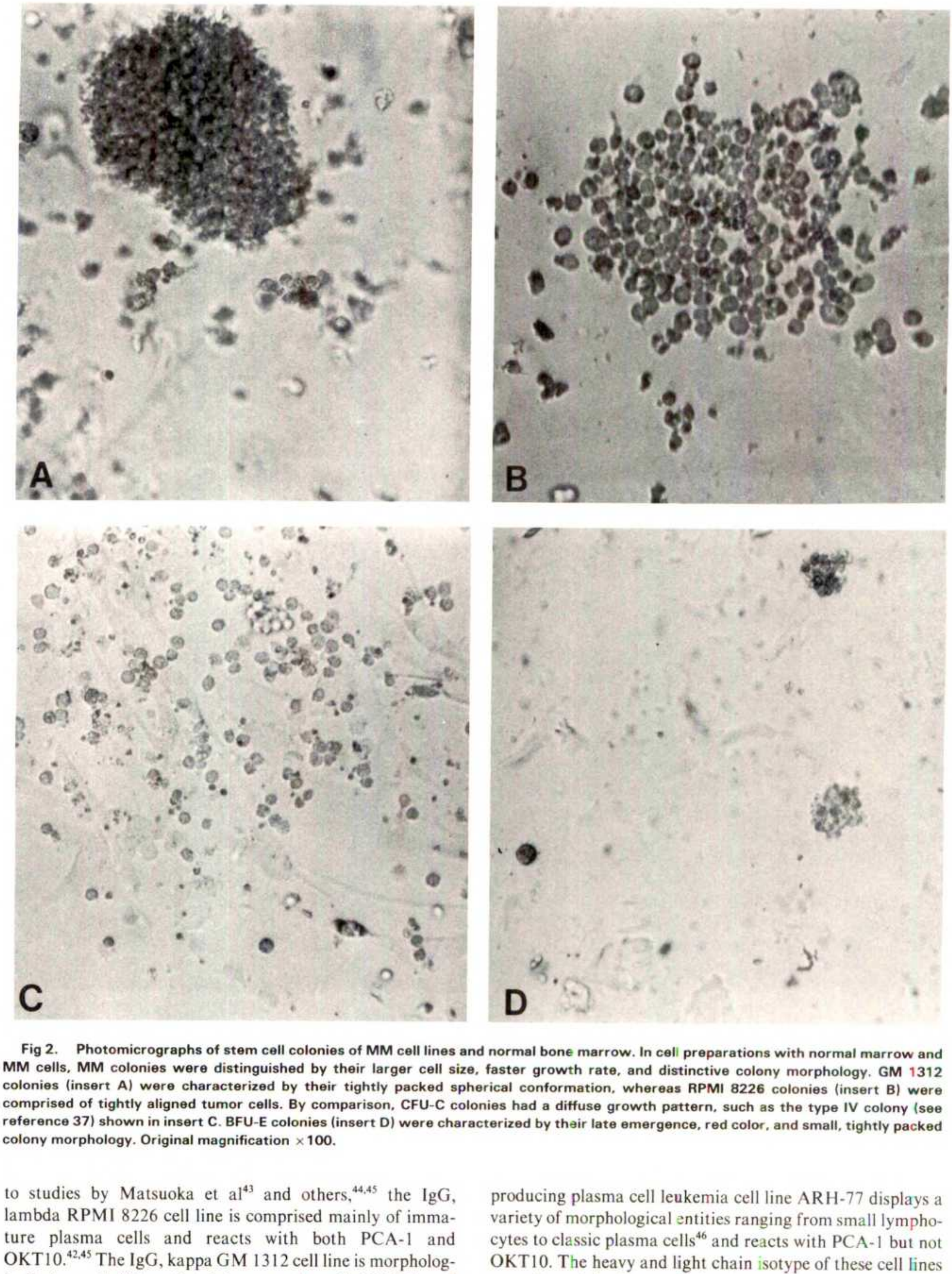

Fig 2. Photomicrographs of stem cell colonies of MM cell lines and normal bone marrow. In cell preparations with normal marrow and MM cells, MM colonies were distinguished by their larger cell size, faster growth rate, and distinctive colony morphology. GM 1312 colonies (insert A) were characterized by their tightly packed spherical conformation, whereas RPMI 8226 colonies (insert B) were comprised of tightly aligned tumor cells. By comparison, CFU-C colonies had a diffuse growth pattern, such as the type IV colony (see reference 37) shown in insert C. BFU-E colonies (insert D) were characterized by their late emergence, red color, and small, tightly packed colony morphology. Original magnification ×100.

to studies by Matsuoka et al[43] and others,[44,45] the IgG, lambda RPMI 8226 cell line is comprised mainly of immature plasma cells and reacts with both PCA-1 and OKT10.[42,45] The IgG, kappa GM 1312 cell line is morphologically less well differentiated, is unreactive with OKT10, and only weakly reactive with PCA-1.[44,45] The IgG kappa-producing plasma cell leukemia cell line ARH-77 displays a variety of morphological entities ranging from small lymphocytes to classic plasma cells[46] and reacts with PCA-1 but not OKT10. The heavy and light chain isotype of these cell lines as well as their immunologic phenotype were confirmed in this laboratory prior to and after culture under clonogenic

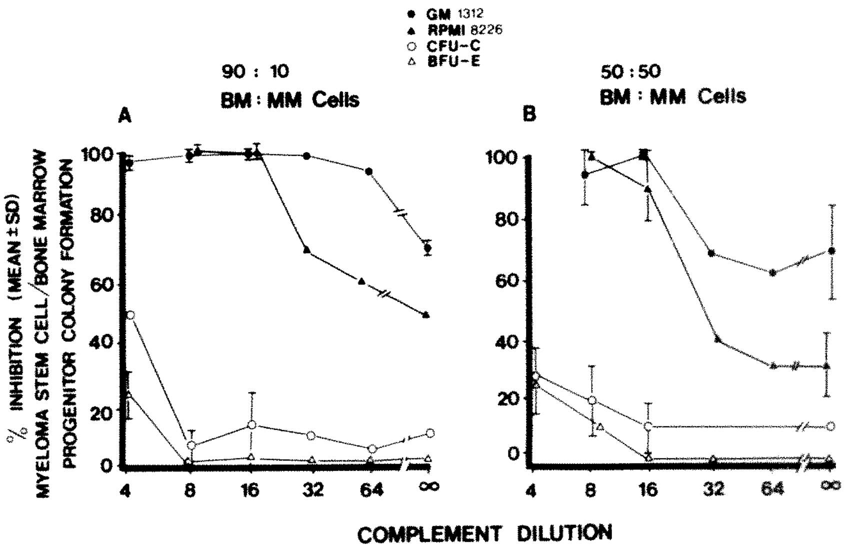

Fig 3. Eradication of myeloma stem cells in mixtures of myeloma and normal marrow cells by MM4 and complement. Normal bone marrow cells were mixed with cells from the MM cell line GM 1312 (●) or RPMI 8226 (▲) in proportions of 90:10 (A) and 50:50 (B). MM clonogenic stem cell colonies in MM4 + C′-treated or untreated samples were enumerated on day 7. Inhibition of normal bone marrow stem cell colonies CFU-C (○) and BFU-E (△) was determined on days 10 and 14, respectively. Value shown represents mean ± SD when based on three or more determinations. Effect of MM4 treatment without C′ is shown as complement dilution at ∞.

stem cell colony forming assay conditions. In this study it was evident that greater than 90% of cells from all three cell lines bound MM4, the majority of which were susceptible to complement-mediated cytolysis. The LD50s of 0.015 to 0.047 mg of MM4 per 10^5 cells were consistent with MoAb concentrations employed for cytolysis of other human tumors.[17-19] Parallel trypan blue exclusion studies indicated that MM4 + C′ was effective in killing >94% of total cells from the PCD cell lines tested.

More significantly, MM4 + C′ consistently abrogated clonogenic stem cell colony growth of the PCD cell lines tested, indicating that the clonogenic stem cell subset expressed the MM4-reactive antigen. As shown in this and our prior study,[27] MM4 alone was effective in inhibiting clonogenic stem cell colony formation of MM cell lines by an as yet undefined mechanism. The inhibitory effect was amplified by subsequent incubation with C′, and efficacy of clonogenic stem cell elimination was maintained in our experimental model of normal bone marrow cell preparations mixed with myeloma cells. MM4 + C′ eliminated 2 logs or more of clonogenic myeloma stem cells both in 90:10 and 50:50 preparations from RPMI 8226, GM 1312, or ARH-77 cell lines. The complement-inhibitory effect of BM cells, also documented previously by other investigators,[17,19] was evident in chromium release data but apparently did not affect the capacity of MM4 + C′ in deleting the clonogenic myeloma stem cell subset. Alternatively, this discrepancy may be related to assay artifacts, such as the resorption of released chromium from the lysed MM cells by normal bone marrow bystanders.

Table 3. Efficacy of MM4 + Complement in Eliminating Multiple Myeloma Clonogenic Stem Cell Colony Formation

	Clonogenic U/mL (Mean ± SD)†		
Cell Lines*	Prior Treatment (C_o)	After Treatment (C_t)	% Reduction‡
RPMI 8226	7.831 ± 3.681 × 10^3	45.47 ± 21.37	99.42
GM 1312	1.639 ± 0.770 × 10^3	18.19 ± 8.549	98.89
ARH-77	7.831 ± 3.681 × 10^3	2.512 ± 1.180	99.97
HS-Sultan	0.982 ± 0.452 × 10^3	1.915 ± 0.900	99.81

*Mixed with normal bone marrow mononuclear cells in the ratio of 90:10 (marrow:myeloma cells).

†Determined by limiting dilution and the Spearman estimate (see "Materials and Methods").

‡% Reduction = $(C_o - C_t)/C_o \times 100\%$.

In an earlier report[27] we found that approximately 70% of the total plasma cells in BM biopsies of MM patients express the MM4-reactive antigen. Our studies on established MM cell lines strongly suggest that the clonogenic stem cell subset from myeloma patients also expresses MM4. Whereas heterogeneity of antigen expression precludes the possibility of completely eradicating the entire myeloma/plasma cell population by MM4-mediated cytolysis, the same treatment can selectively eliminate the clonogenic stem cell subset. Several lines of evidence support the thesis that responsiveness of clonogenic stem cell cultures to cytotoxic or other agents correlate with in vivo therapeutic response.[39,47] In patients with multiple myeloma, Karp et al[48] and others[3,40] have shown that clonogenic colony-forming cells from MM marrow are closely related to kinetically active myeloma cells that proliferate in vivo. Elimination or major reduction of this stem cell subset may limit expansion of the myeloma cell clone with potentially advantageous therapeutic consequences.

Recent studies with leukemia and lymphoma cells have indicated success in purging malignant tumor cells from involved BM with the appropriate MoAbs, thereby providing the option for autologous marrow reconstitution following marrow-ablative chemoradiotherapy.[12-13,16] At present this therapeutic alternative is not available for patients with MM, despite syngeneic marrow transplantation studies suggesting that dose escalation may overcome apparent drug resistance in patients with refractory disease.[15] With the use of alkylating agent-predisone regimens, it is possible to achieve myeloma cell reduction to 10% or less of BM content in some MM patients.[1,8,9] Our experimental model suggests that it may be possible to further reduce the BM myeloma stem cell content by an additional 2 logs or more by incubating with MM4 + C′, thereby potentially achieving a total reduction of 3 logs or more of myeloma stem cells. Under the same experimental conditions, toxicity to CFU-C colonies ranged from 0% to 30%. Such experimental variability, which also has been reported by other investigators utilizing MoAb + C′ for purging of tumor cells,[49,50] may be attributable to background toxicity from C′ alone. Alternatively, we cannot exclude the possibility that the MM4-reactive antigen may be expressed on some normal BM progenitor cells at a low density level that ordinarily goes undetected. Immunofluorescence studies indicated that MM4 reacted weakly with a portion of normal BM cells (16.9% ± 9.2%) at a tenfold higher than usual incubating concentration, whereas reactivity with normal PBM cells remained negative (<1%) under such conditions. A proportion of these BM cells may be susceptible to C′-dependent lysis after exposure to MM4. In addition, MM4 is known to react with normal plasma cells,[27] which are present at low levels in normal marrow. Other studies also have demonstrated highly variable BM recovery (50% or less) in terms of cell number[49] or progenitor cell colonies[51,52] following purging procedures that involve MoAbs unreactive with normal BM. However, patient marrow reconstitution does not appear to be affected by such losses.[50,53] Our approach of myeloma cell depletion by MM4 + C′ thus remains potentially applicable for autologous marrow transplantation.

The results described in this paper deal with an experimental model for purging myeloma cells from bone marrow. The validity of this model has been supported by preliminary studies on BM from four MM patients, in whom we observed significant reduction in myeloma clonogenic stem cell colonies after treatment with MM4 + C′. These initial observations will be extended using BM from additional MM patients. Our data, as well as other studies employing complement,[16-19] indicate that the efficacy of selective tumor cytolysis is largely limited by activity and nonspecific toxicity of the complement source. MM4-immunotoxin conjugates may provide a more efficient mechanism for the elimination of myeloma cells in vitro.[20,54,55]

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Variability in 4-Hydroperoxycyclophosphamide Activity During Clinical Purging for Autologous Bone Marrow Transplantation

By Richard J. Jones, Michael Zuehlsdorf, Scott D. Rowley, John Hilton, George W. Santos,
Lyle L. Sensenbrenner, and O. Michael Colvin

We examined the effects of varying incubation conditions on the in vitro activity of 4-hydroperoxycyclophosphamide (4HC). 4HC activity against CFU-GM and against the K562 tumor cell line decreased with increasing the RBC concentration of the incubation mixture. Increasing the concentration of nucleated bone marrow cells in the incubation mixture also decreased the 4HC activity. Evaluation of 53 consecutive patients undergoing autologous bone marrow transplantation (BMT) revealed that the incubation RBC concentration during clinical purging showed a similar effect on CFU-GM recovery. Aldehyde dehydrogenase content of RBCs and nucleated marrow cells appears to be the cause of the inhibition of 4HC activity. Although there was no difference in individual CFU-GM sensitivity to 4HC among normals, previously treated patients undergoing autologous BMT showed significant variability in CFU-GM sensitivity to 4HC. The combined effects of incubation RBC concentration and individual patient 4HC sensitivity appear to account for most of the variability in CFU-GM recovery and speed of hematologic recovery after clinical purging with 4HC.

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AUTOLOGOUS bone marrow transplantation (BMT) is a useful treatment for resistant acute leukemias, non-Hodgkin's lymphomas, and Hodgkin's disease.[1-6] However, a potential obstacle to the optimal utilization of this procedure is the contamination of the autologous graft with tumor cells, even when the tumor is absent morphologically. Sharkis and coworkers showed that 4HC could eliminate or purge leukemic cells from suspensions of marrow and tumor and still permit hematologic reconstitution.[7] Subsequently, purging with 4HC has undergone extensive preclinical and clinical study.[8-14] At our institution autologous marrow grafts are purged with 100 μg/mL of 4HC at a concentration of 2×10^7 nucleated marrow cells/mL for 30 minutes at 37°C. This dose of 4HC was the highest dose that permitted hematopoietic reconstitution in essentially all patients.[14] However, the time to hematopoietic recovery in these patients varied greatly.

Recently work from our institution showed that the CFU-GM content of 4HC-purged bone marrow grafts predicted the speed of hematopoietic recovery.[15,16] However, the recovery of CFU-GM following clinical 4HC-purging ranged from 0.07% to 23%.[15] The reasons for this 300-fold range in CFU-GM recovery are not all known but may represent differences in 4HC activity resulting from varied incubation conditions, differences in individual hematopoietic progenitor sensitivity, or a combination of both factors. Although serum concentration, nucleated cell concentration, time, and temperature of the 4HC incubation mixture were held constant during clinical purging, the RBC concentration of the incubation mixture varied considerably among patients. Preliminary clinical data from our institution suggested a relationship between the incubation RBC concentration and CFU-GM recovery following bone marrow purging with 4HC. A similar relationship for mafosfamide has also been suggested.[2]

To examine the possible causes of the variable CFU-GM recovery following clinical 4HC purging of autologous marrow grafts, we examined the effects of graded concentrations of RBCs and nucleated marrow cells on the in vitro activity of 4HC against normal human bone marrow and against the K562 myeloid leukemia cell line. We also studied the effects of the incubation RBC concentration during clinical purging with 4HC in 53 patients undergoing autologous bone marrow transplantation (BMT) for the treatment of malignancy. Individual CFU-GM sensitivity to 4HC was also determined for 21 patients receiving autologous transplants.

MATERIALS AND METHODS

Bone marrow cells. Bone marrow cells from healthy volunteers were obtained by aspiration from their posterior iliac crests. Samples of the bone marrow grafts harvested from 53 consecutive patients undergoing autologous bone marrow transplantation for the treatment of malignancy were obtained prior to and after clinical purging with 4HC. All healthy volunteers and patients gave informed consent for study participation as approved by The Johns Hopkins Joint Committee on Clinical Investigation. Mononuclear cells (density <1.078 g/mL) for CFU-GM assays were recovered by Ficoll-Hypaque (FH) density centrifugation.

4HC incubations. The 4HC (prepared by one of the authors, O.M. Colvin) was freshly dissolved prior to use. All clinical purging was performed as previously described.[3,14] Briefly, the nucleated cell buffy coat of the bone marrow graft was incubated with 100 μg/mL of 4HC in TC 199 medium containing 20% autologous plasma at a nucleated marrow cell concentration of 2×10^7 cells/mL for 30 minutes at 37°C. The incubation mixture was rapidly cooled to 4°C and centrifuged at 2900 rpm for ten minutes.

Bone marrow cells from normal donors or cells of the K562 myelogenous leukemia cell line[17] were incubated at concentrations of 5×10^6 cells/mL in enriched McCoy's 5A medium[18] with graded concentrations of 4HC at graded concentrations of autologous RBCs for 30 minutes at 37°C. To remove background red cells from the plates, red cells were lysed by incubating the mixture with 0.87%

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NH_4Cl for ten minutes after washing the cells. Parallel experiments revealed that the NH_4Cl incubation did not alter the CFU-GM recovery. 4HC incubations with various concentrations of mononuclear bone marrow cells, K562 cells, and fetal bovine serum (FBS), and with individual patient serum replacing the FBS, were also performed.

Mononuclear bone marrow cells from 21 autologous bone marrow grafts, obtained prior to clinical purging with 4HC, and cells from 14 healthy volunteers were incubated with graded concentrations of 4HC at concentrations of 2×10^7 cells/mL for 30 minutes at 37°C to determine individual CFU-GM sensitivities to the drug in the absence of RBCs. All experimental 4HC incubations were arrested by the addition of chilled medium, and the cells were washed twice.

Colony assays. CFU-GM assays following the experimental incubations were performed using a modification of the technique described by Pike and Robinson.[18] 2×10^5 mononuclear bone marrow cells/mL were cultured in enriched McCoy's 5A medium containing 0.3% agar and 10% human placental-conditioned medium as a source of colony stimulating factor.[19] CFU-GM assays performed on the clinical autologous marrow grafts and those done on normal marrows to establish individual CFU-GM sensitivity to 4HC were performed as previously described.[20] Briefly, 5×10^4 to 1×10^6 mononuclear marrow cells/mL were cultured in 1.32% methylcellulose containing alpha medium, 5% phytohemagglutinin-stimulated leukocyte-conditioned medium, 1% bovine serum albumin (BSA), 30% fetal bovine serum (FBS), 10^{-6} mol/L methylprednisolone, 10^{-4} mol/L 2-mercaptoethanol, and 1 unit/mL erythropoietin. Clonogenic assays of K562 cells were performed using 0.3% agar, enriched McCoy's 5A medium, and between 200 and 10^4 cells/mL. Parallel experiments have shown that K562 colony growth is linear over this range, with inhibition of growth occurring at K562 cell densities greater than 10^4 cells/mL. One milliliter of the bone marrow or K562 cell mixture was plated in 35-mm petri dishes. The plates were incubated for ten to 14 days in a humidified atmosphere containing 5% CO_2 at 37°C. Assays were performed in quadruplicate, and the plates were scored using an inverted microscope. A colony was considered to be an aggregate of 40 or more cells.

Intracellular 4HC metabolism. Determination of intracellular metabolites of activated cyclophosphamide used 4HC prepared from [^{3}H-side chain] cyclophosphamide (Amersham) by the method of Hohorst et al.[21] Cells were exposed to the radiolabeled 4HC (20 μCi/μmole) at 37° before being rapidly separated from the radioactive medium by centrifuging through oil to give a cell pellet that is extracted by freeze-thawing at neutral pH. A protein-free extract was applied to a reverse phase C_{18} high-pressure liquid chromatography column and eluted with an ion-pairing solvent. Fractions of the eluate were collected and their radioactivity determined by scintillation counting. The intracellular concentration of individual components of the mixture of metabolites was determined from the specific activity of the [^{3}H] 4HC and the intracellular volume of the cells being analyzed.

Statistical evaluations. All the graphed data points from the preclinical studies were the means (±SEM) of two to four separate experiments performed in quadruplicate. The logarithms of percent surviving CFU-GM were plotted against the 4HC incubation concentration. Linear regression analyses (using results of individual experiments rather than means) with calculation of Pearson's correlation coefficients and significance levels were performed. The logarithms of surviving CFU-GM after incubation with 100 μg/mL 4HC were plotted against the respective 4HC incubation-packed RBC volumes for the clinical specimens. An exponential function, $y = be^{ax}$ (y = log of % surviving CFU-GM, x = incubation-packed RBC volume), was chosen as the best fit for the data. Linear regression analysis was employed to estimate the parameters a and b and to yield a correlation coefficient with significance level. The difference in the frequency of an increased CFU-GM sensitivity to 4HC between patients and normal donors was compared by the chi-square test.

RESULTS

The effect of RBC concentration on the activity of 4HC against CFU-GM from healthy donors was assessed. Graded concentrations of 4HC were incubated with 5×10^6 mononuclear marrow cells/mL for 30 minutes at 37°C at incubation-packed RBC volumes of 0%, 1%, 5%, 10%, and 20%. A significant linear relationship was found between the 4HC concentration and the logarithm of CFU-GM survival for each packed RBC volume tested (Fig 1). The 4HC activity against CFU-GM decreased with an increase in the RBC content of the incubation mixture. Similar effects of RBC on 4HC activity against K562 cells were observed (Fig 2). Again there was a significant linear relationship between 4HC concentration and the recovery of K562 clonogenic cells. Adding RBCs to the incubation mixture increased the survival of K562 cells.

The effect of varying the concentration of marrow mononuclear cells in the incubation mixture on 4HC activity was measured. Graded concentrations of 4HC were incubated with 5×10^6, 10×10^6, and 20×10^6 cells/mL for 30 minutes at 37°C. The CFU-GM survival (log) was significantly linearly related to the incubation dose of 4HC at each mononuclear cell concentration tested (Fig 3). The activity of 4HC also decreased with an increase in the concentration of mononuclear cells in the incubation mixture. Increasing

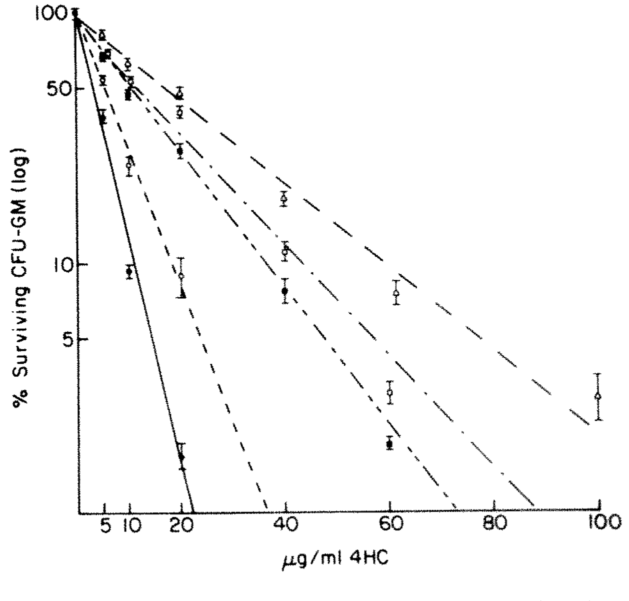

Fig 1. CFU-GM dose response to 4HC as a function of incubation hematocrit: ●——●, 0%; ○- -○, 1%; ■— - - —■, 5%; □— - —□, 10%; △— —△, 20%. The respective regression equations are: $y = 1.98 - 0.091x$ ($n = 46$, $r = -0.98$); $y = 1.99 - 0.057x$ ($n = 36$, $r = -0.95$); $y = 1.98 - 0.027x$ ($n = 52$, $r = -0.98$); $y = 1.94 - 0.022x$ ($n = 69$, $r = -0.94$); $y = 1.98 - 0.017x$ ($n = 62$, $r = -0.97$). All correlation coefficients are significant at $P < 10^{-6}$.

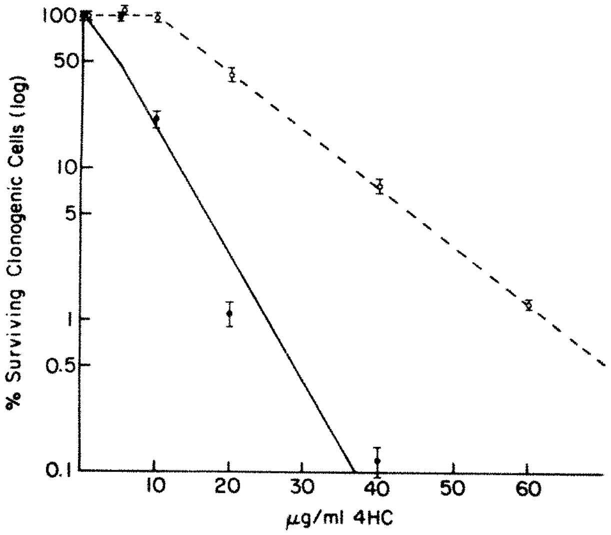

Fig 2. K562 dose response to 4HC as a function of incubation hematocrit; ●—●, 0% (y + 2 = 4.09 − 0.084 ×, n = 44, r = −0.94); ○ - - - ○, 10% (y + 2 = 4.36 − 0.38 × (n = 48, r = −0.98). Both correlation coefficients are significant at $P < 10^{-6}$. The initial shoulders were not included in the linear regression analyses.

the incubation concentration of K562 cells produced similar effects on the 4HC activity against these cells (data not shown).

Evaluation of the relationship between the RBC concentration of the incubation mixture during clinical purging with 4HC and CFU-GM recovery was performed in 53 consecutive patients undergoing autologous BMT. The incubation packed RBC volumes ranged from 4% to 23% with a mean of 8.7%. The CFU-GM recovery after 4HC treatment ranged from 0.01% to 23.8% (mean 1.4%). There was a statistically significant positive correlation between the incubation-packed RBC volume and CFU-GM recovery (Fig 4).

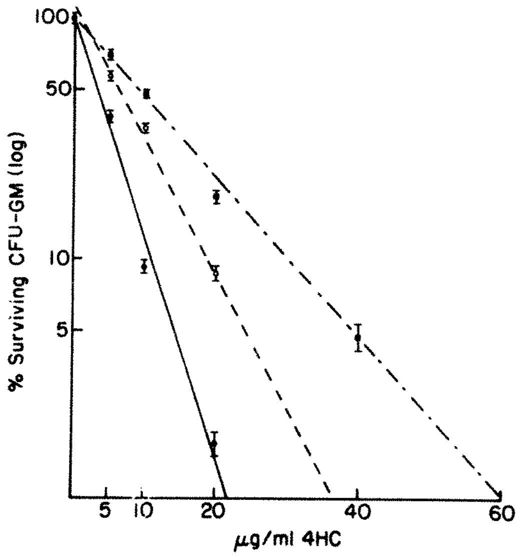

Fig 3. CFU-GM dose response to 4HC as a function of the incubation concentration of mononuclear bone marrow cells; ●—●, 5 × 10^6 cells/ml; ○ - - - ○, 10 × 10^6 cells/ml; ■ — - — ■, 20 × 10^6 cells/mL. The respective regression equations are: y = 1.98 − 0.091 × (n = 46, r = −0.98); y = 2.04 − 0.057 × (n = 40, r = −0.99); y = 2.0 − 0.034 × (n = 39, r = −0.98). All correlation coefficients are significant at $P < 10^{-6}$.

The individual CFU-GM sensitivity to 4HC was tested in 21 of the patients undergoing autologous BMT and 14 normal volunteers. Mononuclear bone marrow cells, obtained from autologous bone marrow grafts prior to clinical purging with 4HC, were incubated with graded concentrations of 4HC at cell concentrations of 2 × 10^7 cells/mL for 30 minutes at 37°C. The individual CFU-GM sensitivity to 4HC was expressed as the slope of the individual dose response obtained by plotting the concentration of 4HC (µg/mL) in the incubation mixture *v* the logarithm of the percent CFU-GM survival. The mean (±SD) 4HC sensitivity for the 14 normals was 0.03 ± 0.008. CFU-GM sensitivity to 4HC was greater than 1 SD above the mean in only one of 14 normal donors, while ten of 21 patients showed CFU-GM sensitivity to 4HC that was greater than 1 SD above the mean (P = .03). In addition, the CFU-GM sensitivity to 4HC was over 4 SD above the mean in two of these ten patients. No patient or normal donor had CFU-GM sensitivity to 4HC that was less than 1 SD below the mean. Differing concentrations of FBS or incubations with serum from different patients did not alter the 4HC sensitivities (data not shown).

The metabolism of 4HC in RBCs was followed by analysis of the intracellular concentrations of activated cyclophosphamide (4-hydroxycyclophosphamide), carboxyphosphamide, and phosphoramide mustard, which together make up 90% of the cyclophosphamide metabolites. Figure 5 indicates the concurrent disappearance of 4-hydroxycyclophosphamide and the appearance of carboxyphosphamide for 10^8 RBCs/mL. This model predicts that 10^8 RBCs/mL (or approximately a 1% incubation-packed RBC volume) would

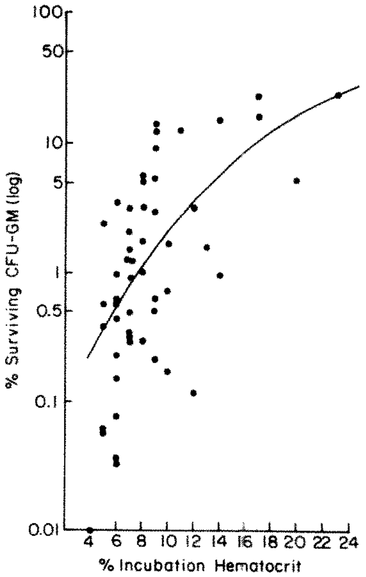

Fig 4. CFU-GM dose response to 4HC from patients undergoing autologous BMT based on incubation hematocrit. Regression equation is $2 - y = 3.54\,e^{-0.075x}$ (n = 53, r = 0.65, p < 0.01).

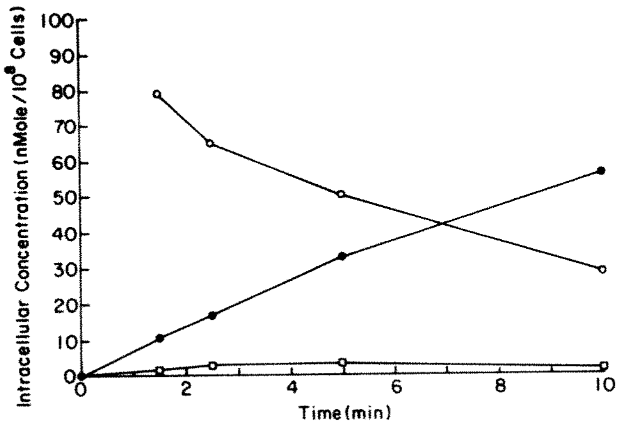

Fig 5. Analysis of cyclophosphamide metabolites in mouse RBCs; O—O, hydroxycyclophosphamide; ●—●, carboxyphosphamide; □—□, phosphoramide mustard. Mouse RBCs (10^8/mL) in RPMI 1640 medium were exposed to 100 μmol/L [^{3}H] 4-hydroperoxycyclophosphamide (20 μCi/μmol) at 37°C. One-milliliter samples were removed at the times indicated and the cells analyzed for cyclophosphamide metabolites as described in the "Methods" section.

metabolize approximately 5 nmol (1.5 μg) of 4HC to inactive carboxyphosphamide during each minute of incubation.

DISCUSSION

We found that variations in the concentration of RBCs or nucleated bone marrow cells in the incubation mixture during purging with 4HC will significantly alter the 4HC activity against CFU-GM. It appears that the actual 4HC effect associated with 100 μg/mL of 4HC incubated with 2×10^7 cells/mL and variable RBC concentrations ranges from that associated with 20 μg/mL to 80 μg/mL of 4HC in the absence of RBCs. This inhibitory effect of the RBCs on 4HC activity also exists toward tumor cells. Therefore a high incubation RBC concentration during clinical purging with 4HC would be expected to shorten hematologic recovery following autologous marrow transplantation but would also likely be associated with decreased tumor kill. Alternatively, a low incubation concentration of red cells should increase tumor kill but would significantly lengthen hematologic recovery. In addition, there is variation in CFU-GM sensitivity to 4HC among individual patients, as also occurs during purging with mafosfamide for autologous BMT.[2] Therefore the variable CFU-GM recoveries, and hence rates of hematologic recovery, seen after marrow purging with 4HC[3,15] result from variable concentrations of RBCs in the incubation mixture and individual patient differences in 4HC sensitivities.

It appears that the decreased 4HC activity seen with increasing the number of RBCs in the incubation mixture is due to the aldehyde dehydrogenase content of these cells. RBCs have been previously shown to contain measurable aldehyde dehydrogenase activity,[22] and the metabolism of 4HC in RBCs, as shown in Fig 5, is characteristic of a cell whose cyclophosphamide metabolism is dominated by aldehyde dehydrogenase.[23] The very rapid consumption of activated cyclophosphamide is consistent with the reduced in vitro activity of 4HC that occurs with increasing the RBC concentration. It is likely that the decreased activity of 4HC seen with increasing the number of mononuclear marrow cells or K562 cells in the incubation mixture is also due to aldehyde dehydrogenase activity in these cells. Bone marrow precursor cells[24] and K562 cells[25] have been shown to contain substantial amounts of this enzyme activity. It also has been shown that L1210 cells can develop resistance to cyclophosphamide through increased aldehyde dehydrogenase activity.[23]

Bone marrow progenitors from patients previously treated with chemotherapeutic agents are frequently more sensitive to 4HC than progenitors from normal donors. This effect of previous therapy on drug sensitivity is the opposite of what occurs with malignant tumors.[26] The reasons for the development of increased CFU-GM sensitivity to 4HC in previously treated patients undergoing autologous BMT are unknown. Radiation recall of normal tissues is another example of prior therapy causing normal tissues to become more sensitive to later chemotherapy.[27]

Two different culture conditions for CFU-GM assays were used in this study. The specimens from the studies shown in Figs 1 and 3 were assayed using standard conditions as described by Pike and Robinson.[18] The specimens from the autologous marrow grafts, and from normals used to determine individual patient CFU-GM sensitivity to 4HC, were assayed using an enriched methylcellulose culture system.[20] We found that the CFU-GM sensitivity to 4HC was not altered by the different culture conditions. The slope of dose response for 2×10^7 mononuclear bone marrow cells from normals incubated with 4HC for 30 minutes at 37°C was 0.03 using either the standard culture conditions (Fig 3) or the enriched methylcellulose culture system. We did find, however, that the enriched culture conditions allowed nearly a tenfold increase in CFU-GM cloning efficiency (data not shown).

It is important to standardize incubation conditions during clinical marrow purging. As we have shown, RBCs contaminating the incubation mixture for purging with 4HC are not inert. The mononuclear cell concentration, duration, and temperature of the incubation all affect the progenitor survival following treatment with 4HC. We have recently begun to adjust all incubation-packed red cell volumes to 7% during clinical purging with 4HC. This incubation RBC concentration was the value predicted by the regression line in Fig 4 for a CFU-GM recovery of 1.4%, the mean recovery seen in our study. Because of differences in CFU-GM sensitivity to mafosfamide between patients, Gorin et al[2] have suggested that the purging dose of this agent be individualized to ensure consistent engraftment. We have chosen, however, not to individualize the dosage of 4HC used in purging. We have found that just standardizing the RBC concentration of the incubation mixture prevents markedly prolonged granulocytopenia following autologous marrow transplantation. In addition, individualizing the dosage of a purging agent based on patient CFU-GM sensitivity does not take into account the potentially more important differences in individual tumor sensitivity to the purging agent.

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A Specific Assay for Anti-HLA Antibodies: Application to Platelet Donor Selection

By Frederick E. Millard, Patricia Tani, and Robert McMillan

Alloimmunization to donor class I HLA antigens represents a major obstacle to successful platelet transfusion therapy. It is desirable to distinguish alloimmunization from nonimmunologic causes of poor platelet survival to assess the need for HLA-matched, single-donor platelets. We describe a new in vitro assay for anti-HLA antibodies and report its application to the problem of platelet crossmatching. In contrast to previously described crossmatch techniques, the immunobead assay is specific for anti-HLA antibodies. The assay was used to evaluate 51 single-donor platelet transfusions given to seven patients from 35 different donors. Recipient plasma was assayed for antibodies directed against HLA antigens present on donor platelets. A one-hour posttransfusion corrected count increment of ≥7,500 was considered a successful outcome. Twenty-nine of 33 (87.9%) transfusion episodes associated with a negative immunobead assay had successful outcomes. The four unsuccessful transfusions were associated with potential nonimmunologic causes of poor platelet survival. Only two of 18 (11.1%) episodes associated with a positive assay had successful outcomes. Only one unsuccessful transfusion episode was associated with a negative immunobead assay and a positive radiolabeled antiglobulin test result, which suggested that isolated alloantibodies to antigens other than class I HLA antigens are not a common cause of platelet refractoriness. Platelets stored in suspension at 4°C or frozen in liquid nitrogen were found suitable for crossmatch testing.

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SINCE ITS BEGINNINGS over three quarters of a century ago,[1] platelet transfusion therapy has played an increasingly important role in the supportive care of patients with bone marrow failure. A significant proportion of recipients become refractory to repeated transfusions from random donors due to alloantibodies to foreign class I HLA antigens present on the platelet surface.[2,3] Yankee et al first demonstrated that HLA-matched platelets from single donors frequently give good increments in this situation.[4,5] Unfortunately, HLA-matched donors are available for only a minority of alloimmunized patients, and the response to partially matched donors is less predictable.[6,7] The recognition of cross-reacting groups and the differential expression of certain HLA antigens (eg, B12) on platelets has facilitated educated guessing during donor selection[8-11]; however, a means of selecting appropriate donors without resorting to trial and error would be advantageous. A variety of approaches to platelet crossmatching have been investigated, but none has proved sufficiently convenient and reliable to become generally accepted.[12-23] In this report, we describe and characterize a new method of identifying anti-HLA alloantibodies. The method was used to evaluate 51 transfusion episodes in seven patients. A platelet radioactive antiglobulin test (RAGT) was performed simultaneously and the results compared with the immunobead results.

MATERIALS AND METHODS

Patient and Donor Population

Fifty-one transfusion episodes involving seven patients with bone marrow failure were studied (Table 1). All were refractory to random donor platelets before entry into the study. Transfusion episodes associated with fever, infection, or splenomegaly were not routinely excluded if good increments from any donor were seen within 24 hours. No patient demonstrated evidence of disseminated intravascular coagulation during the study period. Patient no. 1 was dropped from the study when he became refractory to all donors a few days before his death, associated with adult respiratory distress syndrome (ARDS) and mechanical ventilation. Patient no. 2 was dropped from the study when he became persistently febrile and refractory to all donors. He was subsequently determined to have a disseminated fungal infection. Donors were selected from family members when possible (n = 16), otherwise from paid community volunteers (n = 35). HLA matching was as follows: A, 9; B/B2X, 5; C, 19; D, 8; unknown, related, 7; unknown, unrelated, 3.[9]

Assessment of Transfusion Outcome

Each transfusion episode was evaluated by computing the one-hour corrected count increment (CCI) from the following standard formula: (one-hour postcount − pretransfusion count) × body surface area in square meters/number of platelets transfused/10^{11} = 1-hour CCI. When available, a later CCI (8 to 24 hours) was also calculated. A successful outcome was defined as a one-hour CCI of >7,500. In one instance, a one-hour CCI of 7,289 with a six-hour CCI of 4,252 was considered "successful" when a transfusion from an HLA-matched sibling given less than 24 hours later gave a one-hour CCI of 8,391 and six-hour CCI of 2,670. In two instances where one-hour platelet postcounts were not available, an eight-hour CCI <0 not associated with overt nonimmunologic causes of poor platelet survival was considered evidence of an unsuccessful transfusion outcome.

Murine Monoclonal Antibodies

HB-43 is an IgGκ antibody derived from the 1410K67 cell line, which recognizes the Fc portion of human IgG. HB-95 is an IgG2

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Table 1. Patient Characteristics

Patient	Age/Sex	Diagnosis	Evaluable Transfusions	Number of Different Donors
1	42/M	AA, allo-BMT	22	14
2	32/M	ALL, auto-BMT	2	2
3	21/M	AML	5	3
4	36/F	AML	2	2
5	41/M	AML	1	1
6	71/M	AML	17	11
7	57/M	Secondary MDA → AML	2	2
Totals			51	35

Abbreviatons: AA, aplastic anemia; allo-BMT, allogeneic bone marrow transplantation; auto-BMT, autologous bone marrow transplantation; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.

antibody derived from the W6/32 cell line, which recognizes a monomorphic epitope on the heavy chain of human HLA-A, -B, and -C antigens.[24] The intact heterodimer is required for full expression of the epitope, although HB-95 binds weakly to isolated heavy chains.[25] Both cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were used to produce ascites in Pristane-primed BALB/c mice from which antibody was purified by protein A affinity chromatography. ^{125}I labeling was accomplished by the chloramine-T procedure.[26]

Preparation and Storage of Platelets

Normal platelets. Blood from normal adult volunteers was drawn into EDTA and platelet-rich plasma separated by centrifugation at 400 g for ten minutes. The platelets were pelleted by centrifugation at 1,000 g for 15 minutes, washed four times with 1.0 mL of 0.05 mol/L citrate buffer (14.7 g sodium citrate, 5.9 g sodium chloride, and 25.0 g dextrose in 1,000 mL distilled H_2O, all of which was adjusted to pH 6.2 with 0.05 mol/L citric acid), and resuspended in citrate buffer to approximately 10^9/mL. Platelets were used fresh or stored in suspension at 4°C for 24 to 48 hours.

Donor platelets. Approximately 3 mL of platelet concentrate was obtained at the end of each pheresis procedure. This was divided into 1-mL aliquots and centrifuged at 750 g for 90 seconds to remove red cells. The platelets were centrifuged at 3,000 g for five minutes and washed four times. The platelet concentrate could be kept for 24 to 48 hours at 4°C before washing without affecting assay results.

Platelet storage. To assess the effect of storage on the immunobead assay, 1.0-mL platelet aliquots (1.3×10^9) from two normal donors were stored both in suspension at 4°C and by freezing in liquid nitrogen and then tested at intervals of 1, 6, 15, 30, 60, and 120 days against two control plasmas and two plasmas known to contain high-titer anti-HLA antibodies. Platelets to be frozen were suspended in autologous plasma with 5% dimethylsulfoxide in plastic tubes, frozen at −70°C overnight, then kept in liquid nitrogen. After thawing in a 37°C water bath, they were washed twice, resuspended in approximately 1 mL of citrate buffer, and counted. Parallel aliquots were suspended in normal saline with 0.02% sodium azide in plastic tubes and kept at 4°C. Before assay, the platelets were resuspended by pipetting, washed twice, and counted.

Preparation and Storage of Plasma and Serum

All assays were conducted on plasma, except as noted. Plasma was separated from EDTA-anticoagulated whole blood by centrifugation at 1,000 g for 15 minutes and kept in polypropylene tubes. Test plasma was obtained from recipients before transfusion and after any preceding transfusions whenever possible. In no case was test plasma obtained after the transfusion of interest. Positive control plasmas were obtained from multiply transfused individuals with known high-titer, broad-spectrum anti-HLA antibodies as demonstrated with the immunobead assay against a panel of normal platelets. Negative control plasmas were obtained from normal volunteers with no history of transfusion or pregnancy. Both positive and negative control plasmas were ultracentrifuged at 100,000 rpm for one hour. All plasmas were used fresh or stored frozen at −70°C. Repeated freezing and thawing was avoided.

Sera were prepared from a small number of positive and negative control plasmas by incubation in glass tubes in the presence of $CaCl_2$ and thrombin.

Immunobead Assay

The assay is depicted schematically in Fig 1. Aliquots of 10^8 donor platelets were sensitized by incubation with 100 mL of test or control plasma for one hour in plastic tubes. The sensitized platelets were washed three times, resuspended in 900 mL of citrate buffer, and solubilized by adding 100 mL of 10% Triton X-100. If the assay was to be completed the same day, the lysate was centrifuged at 3,000 g for five minutes and the supernatant transferred to 15-mL polypropylene tubes. The lysate could be frozen at −20°C after solubilization and the assay completed at a later date without affecting the results.

Immunobeads were prepared by incubating ¼-in polystyrene beads (#0023804, Pierce Chemical Co, Rockford, IL) with antihuman IgG (HB-43, 10 μg/bead) in 0.5 mL per bead of 0.01 mol/L $NaHCO_3$ buffer (pH 8.5) for one hour on a rotator. After four washes with 1.0 mL phosphate-buffered saline (PBS) Tween, they were incubated for an additional hour with 2% bovine serum albumin (1 mL/bead) to block residual binding sites. After four more washes, one bead was added to each tube containing platelet lysate and incubated with constant agitation for one hour. During this incubation, IgG alloantibodies that had been bound to the platelet before solubilization attach via the Fc portion to the HB-43 bound to the bead. The class I HLA antigen remains bound to the Fab portion of the alloantibody after solubilization and is thus bound indirectly to the bead. The beads were washed four more times in PBS-Tween, and incubated for one hour with 400,000 cpm of ^{125}I-labeled monoclonal anti-HLA antibody (HB-95) in 1 mL PBS-Tween. After four final washes, the beads were dried on a paper towel and counted in a Beckman gamma counter (Beckman Instruments, Inc, Fullerton, CA). All incubations were carried out at room temperature.

The test of interest was run in triplicate along with duplicates of one positive and two negative controls. One technician could run 24 simultaneous assays in approximately six hours.

RAGT

The RAGT was performed by a modification of the method of LoBuglio et al.[27] Briefly, 10^8 washed platelets were sensitized by incubation with 100 mL of plasma for one hour at room temperature and then washed three times in citrate buffer. A 3×10^7-aliquot was incubated for 30 minutes with 300,000 cpm of ^{125}I-labeled antihu-

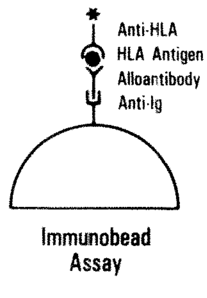

Fig 1. Schematic representation of the antigen-antibody "sandwich" that remains fixed to the polystyrene bead at the end of the assay. The anti-IgG is HB-43 and the anti-HLA, HB-95.

man IgG (HB-43). Triplicate 50-mL aliquots containing 6 × 10^6 platelets were layered over 200 mL of 30% sucrose in 400-μL plastic microcentrifuge tubes with tapered tips and centrifuged at 11,000 g for five minutes. After quick-freezing at −70°C, the tips containing the platelet button were removed and counted in a Beckman gamma counter.

Statistics

The overall predictive values of the immunobead and RAGT assays were compared with the binomial proportions test.

RESULTS

Characterization of the Immunobead Assay

Triplicate assays using 12 negative control plasmas against the same normal platelets gave a mean percent variation of 8.7% ± 9.0% (1 SD). Absolute values of negative controls generally fell in the range of 80 to 300 cpm. A single negative control plasma was also tested against the same platelets ten times and gave a mean percent variation of 6.3% ± 6.6%. Assays run using citrate buffer in place of plasma gave results comparable to negative controls.

To assess the possibility that the assay might recognize antiplatelet antibodies directed against non-HLA antigens, plasmas from 18 patients with the clinical syndrome of idiopathic thrombocytopenic purpura were tested against normal platelets. Seven of these had plasma autoantibodies to the glycoprotein (GP) IIb/IIIa complex, two had autoantibodies to glycoprotein Ib, and the remainder gave negative results for both these autoantibodies. Three plasmas gave positive results in the immunobead assay, two with GP IIb/IIIa autoantibodies and one with a strong antiplatelet autoantibody without demonstrable specificity. All three patients had a history of pregnancy and/or transfusion. Of the negatives, eight had a history of pregnancy or transfusion, two did not, and in five cases these data were not available.

Seven positive and seven negative controls were run with serum and compared with results using plasma. No significant difference was noted between negative controls. The absolute value of serum-positive controls averaged 60% of plasma values. No positives were missed by using serum.

Interpretation of Assay Results

The mean test result was divided by the mean of the negative controls to give a test/control ratio (TCR). Experiments with 12 normals gave a mean normal TCR of 1.00 ± 0.14 (1 SD). A TCR of <1.43 was initially defined as negative and included 3 SD. In practice, TCRs between 1.43 and 3.0 were associated with successful transfusion outcomes in five of six cases. This was interpreted as evidence that the immunobead assay can detect levels of alloantibody below that necessary to significantly affect platelet survival. That this was not due to random variation is supported by the observation that all nine A match transfusions were associated with TCRs <1.43. For clinical purposes, therefore, a TCR of ≤ 3.0 was considered negative and predictive of a successful transfusion outcome.

A TCR of ≤ 2.0 was empirically defined as negative for the RAGT by similar retrospective data analysis.

Correlation With Transfusion Outcome

Each evaluable transfusion episode was categorized according to whether the immunobead assay was positive (TCR, >3.0) or negative (TCR $\leq$ 3.0) and the outcome successful (one-hour CCI, $\geq 7{,}500$) or unsuccessful (one-hour CCI, $<7{,}500$). The data for both the immunobead assay and the RAGT are summarized in Table 2. The immunobead assay was positive in 18 episodes; 16 of 18 (88.9%) had unsuccessful outcomes. In 33 cases the assay was negative; 29 of 33 (87.9%) had successful outcomes. The mean one-hour CCI was 2,199 for assay-positive episodes and 13,072 for assay-negative episodes.

Twenty-one episodes were RAGT-positive; 15 of 21 (71.4%) had an unsuccessful outcome. Of the 30 RAGT-negative episodes, 25 of 30 (83.3%) had a successful outcome. The mean one-hour CCI was 3,897 for RAGT-positive episodes and 11,118 for RAGT-negative episodes.

Thus, the immunobead assay correctly predicted the outcome of 45 of 51 (88.2%) transfusion episodes, whereas the RAGT was correct in 40 of 51 (78.4%) episodes. The difference was not statistically significant ($P = .14$).

In a single instance (involving patient 1), a patient-donor combination initially associated with a positive immunobead assay and an unsuccessful outcome gave a negative assay and a successful outcome 1 month later. The initial transfusion was given just before preparation for bone marrow transplantation with high-dose cyclophosphamide and total nodal irradiation, which suggests that the alloantibody-producing lymphocytes were ablated, after which preformed antibody disappeared.

Figure 2 displays a plot of TCR *v* one-hour CCI that demonstrates the definition between positive and negative results.

Misclassified Transfusion Episodes

Of the six episodes misclassified by the immunobead assay, only one was correctly classified by the RAGT. Six of the 11 episodes misclassified by the RAGT were correctly classified by the immunobead assay. In five instances, both tests gave misleading results.

In the first immunobead assay–positive, successful outcome episode (patient no. 1), the TCR was 5.3 and the one-hour CCI, 16,897. The six-hour platelet postcount had fallen to below the pretransfusion level. The second case

Table 2. Correlation of Immunobead and RAGT Results With Transfusion Outcome

	Assay-Positive		Assay-Negative	
	CCI < 7,500	CCI > 7,500	CCI < 7,500	CCI > 7,500
Immunobead	16/18 (88.9%)	2/18 (11.1%)	4/33 (12.1%)	29/33 (87.9%)
RAGT	15/21 (71.4%)	6/21 (28.6%)	5/30 (16.7%)	25/30 (83.3%)

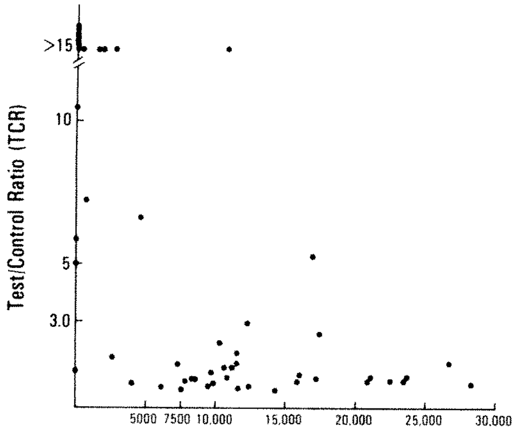

Fig 2. Plot of TCR *v* one-hour CCI for 51 transfusion episodes. A relatively sharp definition between positive and negative assays is seen.

(patient no. 6) had a TCR of 253.7 (the second highest recorded during the study), a one-hour CCI of 10,806, and a 16-hour CCI of 1,965. When the same donor was used again 4 weeks later, the TCR was 183 and the eight-hour CCI, 0. Both patients had unsuccessful outcomes with other donors associated with a positive immunobead assay.

The four assay-negative, unsuccessful outcome cases were all associated with potential non–alloantibody-related causes of poor platelet survival. The first (patient no. 2) was associated with fever and occurred five days before the patient became refractory to all donors and was dropped from the study (see the section Patient and Donor Population). The other three involved patient no. 1: the first occurred when platelets were given simultaneously with a granulocyte transfusion–associated febrile reaction, and the other two occurred after the patient had been intubated for ARDS but before he became refractory to all donors.

Feasibility of Using Stored Platelets

The mean positive control/negative control ratios for both platelet donors at each time interval are displayed in Table 3. The results indicate a significant deterioration in antigenic integrity or availability between one and six days, after which no further major deterioration was detectable through the longest interval tested (120 days). Platelets stored in suspension at 4°C maintained antigenic integrity slightly better than did the frozen platelets, although the difference was not marked. Although the positive controls used here remained markedly positive throughout the study, it is conceivable that plasmas with lower titers of anti-HLA antibody might become negative when tested against stored platelets. There was no significant loss of platelets due to storage during the study period.

Table 3. Immunobead Assay Results Using Platelets Stored Frozen in Liquid Nitrogen and in Suspension at 4°C

	Day of Storage					
Storage Method	1	6	15	30	60	120
Platelet donor no. 1						
4°C	53.8*	26.7	23.9	25.7	21.0	23.2
Liquid N_2	32.6	20.7	23.9	25.3	10.5	23.2
Platelet donor no. 2						
4°C	103.0	33.5	49.3	53.1	48.6	43.3
Liquid N_2	71.5	30.6	35.6	40.5	32.0	44.4

*Data are presented as mean positive control/negative control ratios.

DISCUSSION

The immunobead assay was originally developed to detect autoantibodies against platelet membrane glycoproteins.[28] We have adapted the assay to the study of alloantibodies to class I HLA antigens and applied it to the problem of platelet crossmatching. The results indicate the assay has sufficient predictive value to warrant wider-scale application.

The potential contributions of a reliable crossmatch technique to the conservation of blood bank resources and improved patient care are considerable. The incidence of alloimmunization in patients receiving repeated transfusions is estimated to be at least 50%, with only a small fraction having an HLA-matched related donor available.[29] One large center drawing on an HLA-typed pool of over 2,400 donors found an average of only 1.3 A-matched donors per patient, with 25% of patients having no A or B matches.[7] Most physicians treating alloimmunized patients have considerably smaller donor pools available. The use of partially mismatched platelets greatly enhances the usefulness of small donor pools but requires a reliable crossmatch technique for optimal utilization.

Ideally, prospective donors would have platelets stored when they enter the donor pool. When a patient with a poor response to random donor platelets is encountered, his or her plasma could be assayed against a small panel of normal platelets chosen to represent the common HLA antigens. If negative, it could be assumed that a problem other than alloimmunization against HLA antigens exists and the expense of single-donor platelets avoided. If positive, HLA-matched platelets would be indicated. In the majority of cases where these are not available, the best matches could be pulled from the donor pool and their stored platelets tested, selecting those who test negative. Although our storage study extended only 120 days, other studies suggest that much longer storage periods are feasible.[30-32]

One potential drawback to the immunobead assay is its HLA specificity. Many authors have suggested that alloantibodies to platelet-specific antigens account for some percentage of transfusion failures, usually based on poor response to HLA-identical platelets and/or the lack of demonstrable lymphocytotoxic antibody.[12,18-20,33-35] However, alloantibodies to platelet-specific antigens have been difficult to demonstrate conclusively in this setting, and their true significance remains uncertain. Herman et al used Western blots to study seven patients refractory to HLA-matched platelets and were unable to demonstrate antibody to platelet-specific antigen on the GP IIb/IIIa molecule.[36] They concluded that antibodies to platelet-specific antigens are not commonly present. Our finding of only one in 51 instances of poor transfusion outcome where the RAGT was positive and the immunobead assay negative tends to support this. It seems more likely that unrecognized nonimmunologic factors account for many if not all of these cases. However, it would seem prudent at present to use the RAGT or a similar non–HLA-specific assay to evaluate the presence of antibody to other platelet-associated antigens in cases where the HLA-specific immunobead assay is negative in the face of poor platelet survival.

ACKNOWLEDGMENT

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T Cell Receptor (α, β, γ) Gene Rearrangements and Expression in Normal and Leukemic Large Granular Lymphocytes/Natural Killer Cells

By Pier-Giuseppe Pelicci, Paola Allavena, Milayna Subar, Alessandro Rambaldi, Anna Pirelli, Maria Di Bello, Tiziano Barbui, Daniel M. Knowles II, Riccardo Dalla-Favera, and Alberto Mantovani

The large granular lymphocyte (LGL) population, which effects a natural killer (NK) function, consists of cells whose lineage derivation has not been clearly established on the basis of phenotypic and functional properties. To clarify the relationship of LGL/NK cells to T cells we studied patterns of rearrangement and expression of the T cell receptor (Ti) genes α, β, and γ in normal human LGLs; in CD8$^+$, CD8$^-$, Mol$^+$, and Mol$^-$ LGL subsets; and in 17 cases of leukemic LGL proliferations (TγLPD). T_α, T_β, and T_γ genes were not expressed, nor were T_β and T_γ genes rearranged in normal LGLs or LGL subsets. The T_γLPD were divided into two groups. One group (15/17 cases) was characterized as CD3$^+$ and displayed Ti gene rearrangements. Seven of these cases were reactive with monoclonal antibody WT31, which suggested expression of an α/β heterodimer on the cell surface. The other group (2/17 cases) was CD3$^-$ with unrearranged Ti genes. These results indicate that the normal LGL/NK population is homogeneous and distinct from the normal T cell population because it does not express, and as a result, cannot effect its immune function through the T cell receptor molecules. Conversely, TγLPDs represent a heterogeneous group of lymphoproliferative diseases within which the CD3$^-$, Ti$^-$ cases most likely represent the neoplastic counterpart of normal LGL cells. The more frequent CD3$^+$ cases may be related to recently described NK-like T cells. The observations that normal LGLs maintain germline T_γ genes and that many CD3$^+$ TγLPD display an α/β heterodimer suggest that a T_γ-containing receptor may not be necessary for NK or NK-like cytotoxicity.

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LARGE GRANULAR LYMPHOCYTES (LGL) are the principal effectors of natural killer (NK) function and antibody-dependent cell-mediated cytotoxicity (ADCC, K cell activity). They function in vivo as effectors of natural resistance against foreign cells, certain infectious agents, and tumor cells.[1,2] The LGL/NK population is heterogeneous and consists of cells sharing phenotypic and functional properties with both T cells and myelomonocytic cells.[1,2] As a result, their precise lineage derivation and mechanisms of immune function have remained unresolved and their place in hematopoietic differentiation pathways unclear.

The relationship of LGL development and function to the T cell lineage can be approached by analyzing T cell receptor gene rearrangements and expression. Two genes, T_α[3,4] and T_β[5,6] code for the complete T cell antigen receptor molecule in thymocytes and in peripheral blood T cells.[7] T_β gene rearrangements precede T_α gene rearrangements[3,8] and occur in all functional T cell subsets early during thymic differentiation.[9-11] A third gene, the T_γ gene, isolated from cytotoxic T cells is also a T cell–specific rearranging gene.[12,13] The T_γ gene is believed to rearrange even earlier in T cell differentiation, before the T_β gene.[14] Thus, T_β and T_γ gene rearrangements represent early and irreversible markers of the T cell lineage, whereas T_β and T_α gene expression correlates with expression of the T cell antigen receptor molecule.

Analyses of T cell receptor gene rearrangement and expression in various normal or neoplastic clonal LGL/NK populations derived from different species have led to a complex set of results that does not provide conclusions as to the lineage derivation or mechanisms of immune function of LGL/NK cells.[15-23] The majority of normal and neoplastic human NK clones[15,16] and all mouse NK clones[17,18] displayed T_β gene rearrangements and variably expressed T_β mRNA; a minority of human NK clones, both normal and neoplastic,[15,16] and all rat NK cell leukemias[19] displayed the germline configuration of the T_β gene and did not express T_β mRNA. Circulating LGL/NK cells freshly isolated from normal individuals display the germline configuration of the T_β and T_γ genes[20,21,23] and express a truncated version (1.0 kilobase [kb]) of the normal T_β mRNA (1.2 kb).[22] These discrepancies may reflect different criteria used for LGL/NK selection, the presence of heterogenous LGL/NK subpopulations, differences between normal and neoplastic LGL/NK cells, and finally, differences between different animal species. Based on these considerations, we comprehensively analyzed the genomic organization and patterns of expression of T cell receptor genes in normal and neoplastic human LGL/NK cells and in NK subpopulations selected according to their expression of T cell or myelomonocytic surface markers. We have extended previous studies on the T_β gene by including studies on T_α gene expression and on T_γ gene rearrangements and expression. Our data indicate that normal LGL/NK cells are ontogenically and functionally distinct from T cells.

MATERIALS AND METHODS

Isolation of Peripheral Blood LGLs, T Lymphocytes, and Thymocytes

Isolation of LGLs. Normal mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of fresh heparinized peripheral blood obtained from healthy donors. The peripheral blood

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mononuclear cells were depleted of monocytes by plastic adherence and passage over a nylon wool column and were then fractioned on a six-step Percoll gradient ranging from 35% to 47.5%.[24] Cells from the low-density fractions (60% to 80% LGLs, 20×10^6 cell/mL) were collected and further depleted of mature T cells by repeated 30-minute incubations with monoclonal antibody (MoAb) OKT3 (diluted 1:100; gift of Dr E. Reinherz, Boston) or MoAb Leu-l (Becton Dickinson, Mountain View, CA) and 60-minute incubations with complement (diluted 1:4, Pel-Freeze Laboratories, Rogers, AR). The purity of each LGL preparation was assessed by morphology, the expression of MoAb-defined cell surface antigens, and in vitro cytotoxic activity (see Table 1). Purified fractions of phenotypically distinct LGL/NK subpopulations were then obtained by column immunoabsorption or cell sorting[25] using MoAbs OKT8 and Mol, which identify phenotypically[26,27] and functionally[28,29] distinct LGL/NK subsets. MoAb OKT8 recognizes the CD8 antigen, which is present on the mature T cell subset commonly associated with suppressor/cytotoxic function[30] and on approximately 30% of LGLs (Table 1).[26] MoAb Mol reacts with the Mol epitope of the C3bi receptor,[31] which is present on monocytes, myeloid cells,[32] and approximately 70% of LGLs (Table 1).[26,27,32]

T lymphocytes. Peripheral blood T lymphocytes were isolated by fractionation of peripheral blood mononuclear cells on a Ficoll-Hypaque density gradient according to their capacity to form E rosettes with *Vibrio cholerae* neuraminidase–treated sheep erythrocytes.[33] The E$^+$ fraction consisted of >95% T cells as determined by CD3 expression. The E$^+$ fraction was incubated with OKT8 and OKT4 MoAbs and complement at 37°C to obtain purified fractions of CD4$^+$ and CD8$^+$ cells, respectively. The resulting cell populations were 95% pure as determined by CD4 and CD8 expression.

Thymocytes. Thymocytes were isolated from portions of thymuses collected during the course of cardiac surgery.

MoAb-defined cell surface markers and morphology. The surface immunophenotype of the isolated cells was determined by indirect immunofluorescence using a Zeiss microscope and/or a FACS IV (Becton Dickinson). The following panel of MoAbs was used: OKT3, OKT6, OKT8, OKT11 (Ortho Diagnostic Systems, Inc, Raritan, NJ, or the gift of Dr Reinherz, Dana-Farber Cancer Institute, Boston); HNK-1 (courtesy of Dr Abo, Birmingham, AL), which is reactive with NK cells and a subset of T cells[34]; B73.1 (courtesy of Dr G. Trinchieri, Philadelphia)[2] and AB8.28 (courtesy of Dr Malavasi, Turin, Italy)[35] both reactive with a structure closely associated with the Fc$_\gamma$ receptor of LGLs; N901, reactive with LGLs and some myeloid precursors (courtesy of Dr J. Griffin, Boston),[36] WT31, reactive with a common epitope of the T cell receptor α/β heterodimer (courtesy of Dr W.J.N. Tax, Amsterdam[37]), Leu-11, reactive with the CD16 antigen; and Leu-7 (Becton Dickinson Monoclonal Center, Inc, Mountain View, CA).

Table 1. Phenotypic and Functional Analysis of LGLs and LGL Subpopulations

	Phenotypic Markers*					Cytotoxicity†	
LGL Population	CD8	CD3	Mo1	HNK1	N901	NK	ADCC
LGL	30	0	70	65	60	42	22
LGL-CD8+	97	0	44	61	ND	36	14
LGL-CD8−	<5	0	44	38	ND	41	16
LGL-Mol+	ND	0	99	46	ND	46	23
LGL-Mol−	31	0	0	3	ND	3	2
T lymphocytes	45	98	0	0	ND	2	1

Abbreviation: ND, not done.

*Numbers represent the percentage of positive cells for each of the MoAb stainings.

†Results are expressed as means and refer to an E:T ratio of 50:1.

Cytotoxicity assays. NK activity was assessed by using the K562 cell line at effector-to-target cell (E:T) ratios ranging from 25:1 to 100:1 in a four-hour in a ^{51}Cr release assay as previously described.[38] For ADCC, ^{51}Cr-labeled TLX9 murine lymphoma cells sensitized with 1:10,000 rabbit antibody were used as targets in a four-hour test.

DNA extraction and Southern blot analysis. DNA was prepared by cell lysis, proteinase K digestion, extraction with phenol, and precipitation with ethanol.[39] Fifteen micrograms of DNA were digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to a nitrocellulose filter, and hybridized and washed according to Southern.[39] Hybridized filters were boiled for five minutes in distilled H_2O before hybridization to a second DNA probe.

RNA extraction and Northern blot analysis. Total cellular RNA was extracted according to the guanidine-isothiocyanate method.[39] Five to 10 μg of purified total RNA were electrophoresed in a 1% formaldehyde–containing agarose gel, transferred to nitrocellulose, hybridized, and washed according to established procedures.[39]

DNA Probes

T_β probe. The T_β probe used in this study was derived from a human T_β cDNA clone (YTJ-2) isolated from the Jurkat-2 T lymphoma cell line (courtesy of Dr Tak Mak).[5] This clone hybridizes to both alleles ($C_{\beta 1}$ and $C_{\beta 2}$) of the constant region and to one or more alleles of the variable regions.[5,40]

T_γ probes. Three different T_γ probes were used in this study. The Ca/Cb and V2 probes were derived from two human T_γ cDNAs. The $F_\gamma 7$ cDNA isolated from the Fro 2.2 T lymphoma cell line represents an aberrantly rearranged T_γ allele.[57] The V2 probe is representative of the variable region of $F_\gamma 7$ cDNA and belongs to the $V_\gamma I$ variable region family.[41] The $T_\gamma 5$ cDNA isolated from a peripheral T cell library represents a truncated T_γ constant region. The Ca/Cb probe represents the first exon of $T_\gamma 5$ cDNA (M. Subar et al, submitted for publication). The pJx2 probe is a genomic probe derived from the G2.0 T_γ genomic phage isolated from a Charon 4A human placental library. It represents a unique *Eco*RI-*Hind*III fragment containing the putative J region of the $F_\gamma 7$ cDNA clone.[57] The positions of these three probes with respect to the $F_\gamma 7$ and $T_\gamma 5$ cDNA clones and the human T_γ gene are schematically represented in Fig 1. DNA fragments were ^{32}P labeled by nick-translation[39] for use as probes.

RESULTS

Analysis of T Cell Receptor (α,β) Gene Rearrangements and Expression in LGL/NK Cells and Subsets

Freshly isolated peripheral blood LGLs display the germline organization of the T_β gene but express a truncated version of T_β mRNA.[20-22] However, the majority of clonal LGL/NK cell populations display T_β gene rearrangements.[15-19] Because T cells invariably display T_β gene rearrangements and expression, these data do not permit an unequivocal understanding of the relationship between LGL/NK cells and T cells. To clarify this issue, we tested the hypothesis that minor LGL/NK subpopulations may display T cell receptor gene rearrangements. This hypothesis is particularly relevant in view of the fact that (*a*) a minor LGL/NK subpopulation (30%, see Table 1) is identifiable by the expression of the T cell–specific CD8 antigen and (*b*) a minor LGL/NK subpopulation carrying T_β gene rear-

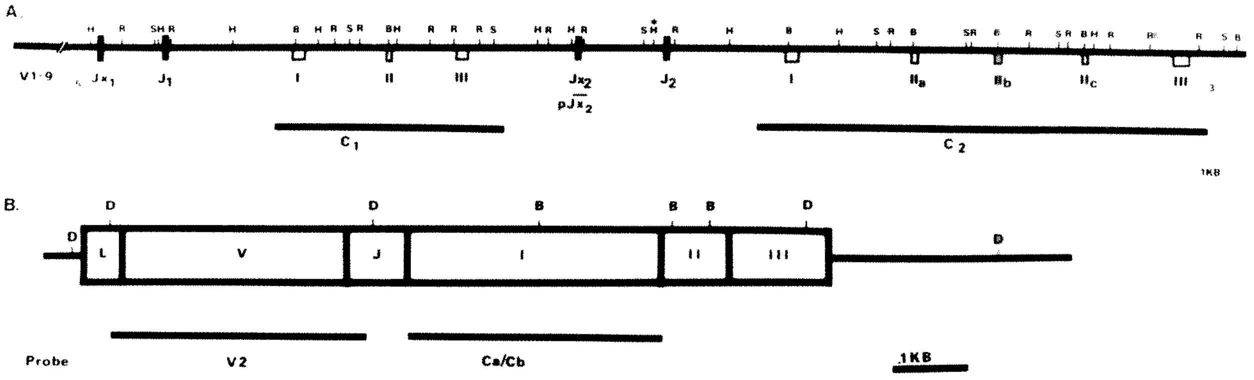

Fig 1. Restriction enzyme maps of the human T_γ locus and of the $F_\gamma 7$ human T_γ cDNA. (A) The two genes (C1, C2) that compose the T_γ constant region locus are indicated. Exons I, II a to c, and III are labeled and indicated by boxes. The pJx2 probe is as shown. (B) The leader (L), variable (V), joining (J) and three constant region exons (CI, CII, CIII) that compose $F_\gamma 7$ cDNA are indicated. The bar below the map indicates a probe representative of the $F_\gamma 7$ variable region (V2). The area equivalent to the Ca/Cb probe derived from the first exon of the $T_\gamma 5$ cDNA is indicated.

rangements could escape analysis when the total LGL/NK population was examined. For these reasons, we analyzed the organization and expression of T cell receptor α and β genes in phenotypically distinct LGL/NK cell subpopulations as well as in unfractionated normal LGL/NK cells.

The genomic organization of the T_β gene was investigated by Southern blot hybridization using a DNA probe specific for the human T_β gene locus. We had previously shown that hybridization of this probe to *Eco*RI-digested DNAs allows the detection of T_β gene rearrangements in both monoclonal and polyclonal T cell populations.[40] The biallelic rearrangement of part of the constant region ($T_\beta C$) can be recognized as a loss of the 12.0-kb *Eco*RI band corresponding to the $C_{\beta 1}$ region in normal, polyclonal human thymocytes and T lymphocytes[40] (see Fig 2, lane T-L). When the total LGL/NK population was studied in this manner, the T_β gene was found in its unrearranged germline configuration. Identical results were obtained in purified $CD8^+$ and $Mo1^+$ LGL/NK subpopulations (Fig 2). In the experiment shown in Fig 2, LGLs were pretreated with anti-CD3 to remove possible conventional T-cell contaminants, but similar results were obtained with anti-Leu-1 (CD5)-treated cells or with the untreated Percoll-purified population. Comparative densitometric analysis of the *Eco*RI 12.0-kb band *v* the other bands in LGL/NK and control cells confirmed that there is no appreciable loss of the $C_{\beta 1}$ allele in the different LGL/NK populations analyzed (data not shown). Therefore, we conclude that the T_β gene remains unrearranged in the vast majority of normal LGL/NK cells.

We next investigated T_α gene expression in freshly isolated LGL/NK cells because a molecular assay to detect T_α gene rearrangements in normal polyclonal cells is not yet available. RNA was extracted from purified LGLs and from the CD8+ LGL/NK subpopulation and analyzed by Northern blot hybridization using a T_α probe (clone PY1.4, a gift from T. Mak). T_α RNA was not detectable in either sample (see Fig 3, middle lane). The same RNA blots were used for hybridization with a human T_β probe (Fig 3, first lane), and no specific hybridization signal was detected. The absence of the CD3 antigen and T_β and T_α gene expression in LGL/NK cells proves that LGL/NK cells do not express an α/β heterodimer on their surface.

Analysis of T_γ Gene Rearrangements and Expression in Normal LGLs, LGL Subpopulations, and T Cells

The lack of T_β gene rearrangements and T_β/T_α gene expression in the majority of LGL/NK cells suggests that the "conventional" T cell antigen receptor is not involved in NK target cell interaction. However, the ontogenic relationship between LGL/NK cells and T cells remains unresolved because T_β and T_α genes are neither rearranged nor expressed in early thymocytes[11] and both early thymocytes

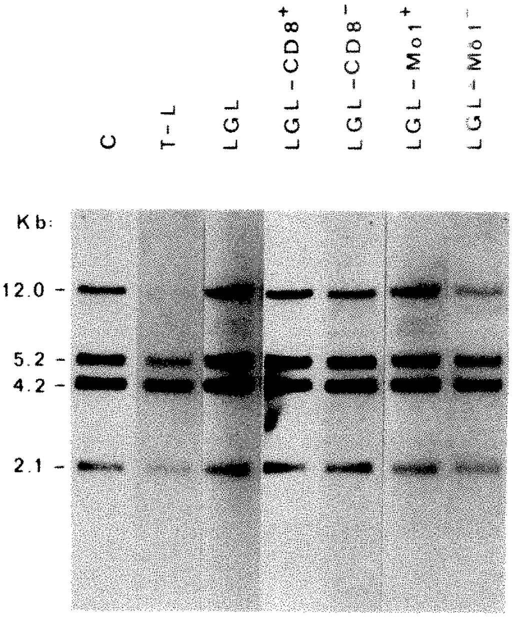

Fig 2. T_β gene rearrangement analysis in fresh uncultured LGLs and LGL subpopulations. DNAs extracted from normal human fibroblasts (lane C), normal human peripheral blood T lymphocytes (lane T-L), and different LGL subpopulations, as indicated, were digested with *Eco*RI restriction enzyme and analyzed by Southern blot hybridization using a T_β gene probe.

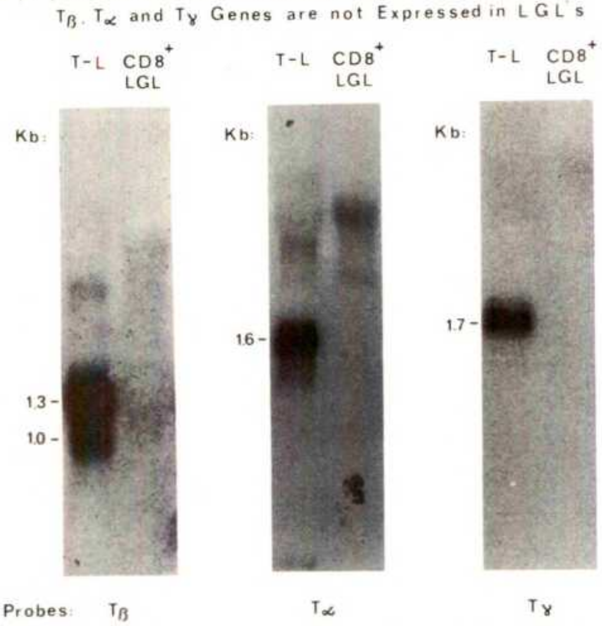

Fig 3. Expression of T_β, T_α, and T_γ RNA in CD8$^+$ LGLs and peripheral blood T lymphocytes. Total cellular RNA was extracted from the CD8$^+$ LGL subpopulation and peripheral blood T lymphocytes (T-L). Northern blots were hybridized to T_β, T_α, and T_γ probes as indicated.

and the majority of LGL/NK cells lack CD3 antigen expression and express the CD8 antigen.[2] We then chose to analyze LGL/NK cells for rearrangement and expression of a third T cell receptor gene, the T_γ gene, which is believed to rearrange early during T cell differentiation before the T_β and T_α genes[14] and may therefore represent a marker for the earliest stages of the thymic differentiation pathway. The T_γ gene has been reported to remain in its germline organization in the total LGL/NK population.[23] However, this analysis was limited to the investigation of T_γ gene rearrangements involving the $T_\gamma J1$ or $T_\gamma J2$ regions. We have recently shown that T_γ rearrangements involving previously unidentified $T_\gamma J$ regions (Jx2; M. Subar et al, submitted for publication) may occur in 25% of T cells and up to 30% of pre-B cells.

Our experimental approach involved Southern blot hybridization analysis of T_γ gene organization in DNAs extracted from the total LGL/NK cell population as well as the CD8$^+$ and CD8$^-$ LGL/NK subpopulations. The results were compared with Southern blot analyses of the T_γ gene organization in DNAs extracted from normal polyclonal T cell populations. The assay for identification of different patterns of T_γ gene rearrangements was based upon results of the analysis of T_γ gene rearrangements in a large variety of clonal T cell populations (M. Subar et al, submitted for publication). A diminution in the intensity of the 5.5-kb *Eco*RI band hybridizing to a T_γ first-exon probe (Ca/Cb) in all polyclonal T cells (see Fig 4A) corresponds to deletions of the $T_\gamma C1$ locus, which are constantly associated with rearrangements of the $T_\gamma C2$ locus (M. Subar et al, submitted for publication). The appearance of a 7.5-kb *Sst*I band hybridizing to the T_γ variable region probe (see Fig 4B) reflects rearrangements involving a member of this variable region family and is seen in polyclonal T cells as in the majority of clonal T cell populations (M. Subar et al, submitted for publication). The appearance of a few faintly hybridizing, Jx2-rearranged bands corresponds to $T_\gamma C1$ rearrangements (see Fig 4C).

The T_γ gene was present in its germline configuration in all DNAs extracted from polyclonal LGL/NK cells and the CD8$^+$ and CD8$^-$ LGL/NK subpopulations. Representative Southern blots for the CD8$^+$ and CD8$^-$ LGL/NK DNAs are shown in Fig 4. We also analyzed the expression of the T_γ gene in the total LGL/NK population and in LGL/NK subsets. No specific hybridization signal was detected in any of the samples (for representative results, see Fig 3, right lane). We conclude that LGL/NK cells and CD8$^+$ and CD8$^-$ LGL/NK cell subpopulations are clearly distinct from both mature and immature T cells with respect to the organization of the T_γ gene and that the T_γ gene is not rearranged or expressed in the majority of, if not all, LGL/NK cells.

Gene Rearrangements and Expression Analysis of T Cell Receptor Genes in Human LGL/NK-Derived Leukemias

An approach commonly used to investigate properties of a normal cell population is to analyze its clonally expanded neoplastic counterpart. This is based on the concept that malignant transformation results in freezing the target cell at a given stage in its normal differentiation pathway, which

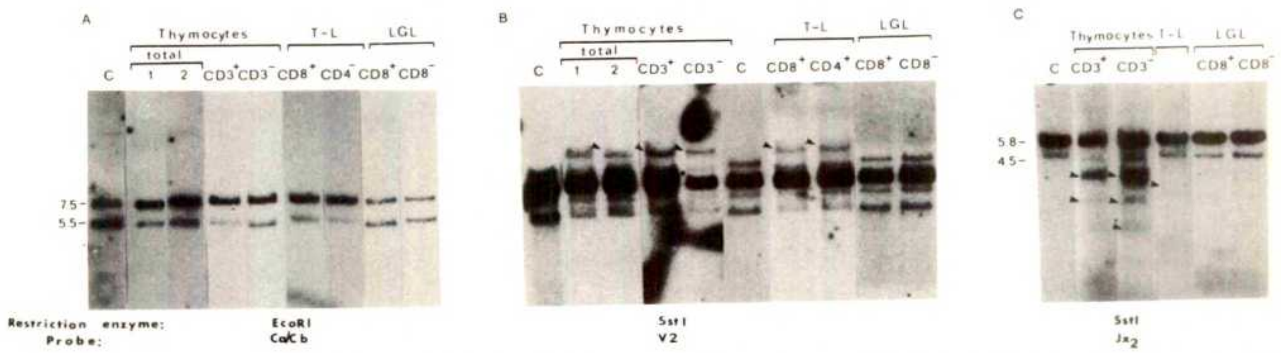

Fig 4. T_γ gene rearrangement analysis in normal T cells and LGLs. DNAs were extracted from different cell populations, digested with the indicated restriction enzymes, and hybridized to the indicated T_γ DNA probes. Arrows indicate rearranged bands. Lane C, human fibroblast DNA.

Table 2. Phenotypic and Immunogenotypic Analysis of TγLPDs

No. Cases	CD3 Antigen Expression*	T_β Gene†	T_γ Gene‡
2	–	G	G
12	+	R	R ($T_{\gamma 2}$)
3	+	R	R ($T_{\gamma 1}$)

*+ represents >20% positive cells after staining with OKT3 MoAb.

†G, germline; R, rearranged.

‡The material in parentheses indicates the occurrence of $T_\gamma C1$ or $T_\gamma C2$ rearrangements.

Table 3. Phenotypic and Functional Analysis of TγLPD Mononuclear Cells

Case No.	Phenotypic Markers*				Cytoxicity‡	
	WT31	CD3	Leu-7	CD16	NK	ADCC
4	60	62	33	50	69	53
7	95	94	83	44	13	40
8	76	90	63	11	5	28
9	77	90	42	29	14	58
11	85	78	55	25	37	25
14	51	47	ND	50	61	49
15	56	81	12	55	29	ND

*Results are expressed as percentages of positive cells.

†Results are expressed as means and refer to an E:T ratio of 50:1.

results in a uniform phenotype for the neoplastic cell population. For this reason, we analyzed the genomic organization of the T cell receptor genes β and γ in a collection of 17 LGL leukemias, so-called Tγ lymphoproliferative disorders (TγLPD).[42]

The principal immunophenotypic, functional, and immunogenotypic properties of 12 of these 17 cases of TγLPD have been reported in detail.[16] The five additional cases of TγLPD shown in this study displayed similar phenotypic and functional features including CD2, HNK-1, AB8.28, and B73.1 expression; Fc_γ receptors identified by rosetting technique; and ADCC effector function (data not shown). Surface markers CD3, CD8, OKM1, and N901 were detected in the LGLs isolated from some but not all cases (data not shown). In particular, two cases did not express the CD3 antigen (Table 2). LGLs from all TγLPD patients had high ADCC, whereas high NK activity was only detected in six cases (Table 3).[16] Fifteen cases displayed clonal rearrangements of the T_β gene, and two cases displayed the germline configuration of the T_β gene upon *Eco*RI and/or *Bam*HI digestion and hybridization to a T_β probe (data not shown). The two cases displaying the T_β gene germline organization were the same two cases that lacked the CD3 antigen.

All 17 cases of TγLPD were analyzed for the organization of the T_γ gene. Representative Southern blots of this analysis are shown in Fig 5, and a synthesis of the results is reported in Table 2. We detected T_γ gene rearrangements in 15 of 17 TγLPD cases tested using the same combination of restriction enzymes and probes described for the analysis of T_γ gene

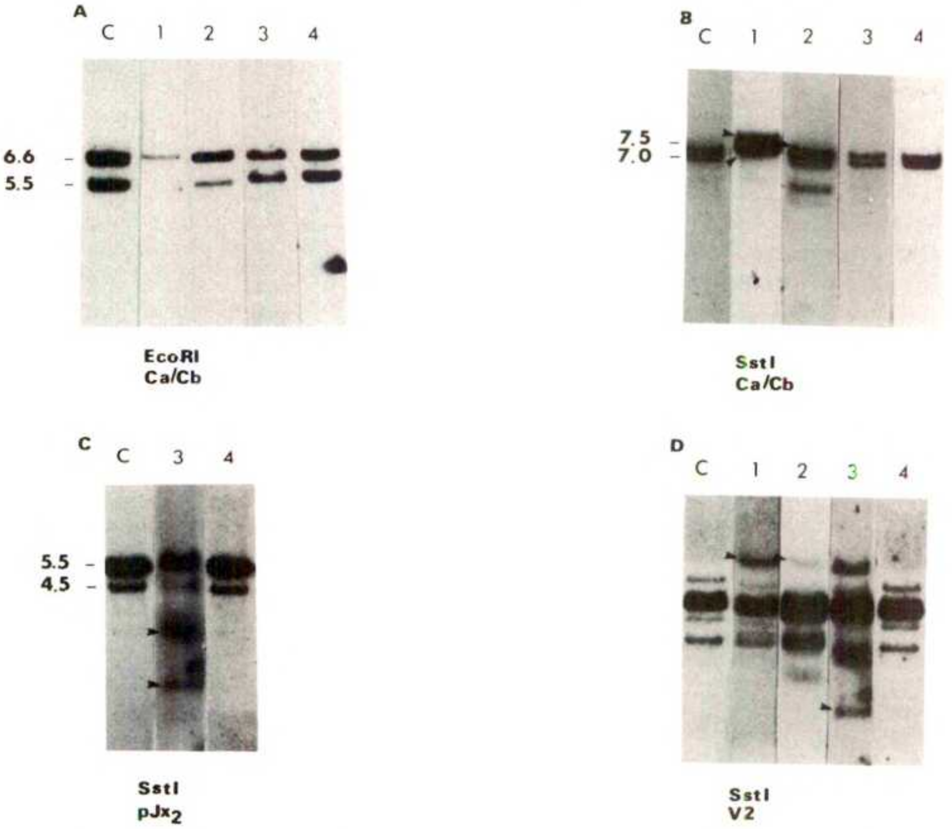

Fig 5. T_γ gene rearrangement analysis of T_γLPDs. DNAs were extracted from leukemic LGLs of different TγLPD patients, digested with the indicated restriction enzyme, and hybridized to the indicated probe. Arrows indicate rearranged bands. Lane C, human fibroblast DNA.

rearrangements in polyclonal T cells. In 12 of 17 TγLPD DNAs tested, the Ca/Cb hybridizing 5.5-kb *Eco*RI band corresponding to exon I of the $T_{\gamma}C1$ gene appeared deleted or fainter than the respective control bands, which suggested a deletion of that DNA region on one or both alleles (see Fig 5A, lanes 1 and 2, for representative results displaying the biallelic or monoallelic deletion of the $T_{\gamma}C1$ gene, respectively). The same DNAs were digested with the *Sst*I restriction enzyme and hybridized to the Ca/Cb probe to detect rearrangements involving the J1 or J2 regions (for a map of the T_{γ} locus, see Fig 1A). One or two rearranged bands were detected in each of these 12 cases (see Fig 5B, lanes 1 and 2). Hybridization of the same blots to a T_{γ} variable region probe (V2) showed the appearance of one or two rearranged bands (Fig 5D, lanes 1 and 2) with at least one rearranged band containing both V2 and Ca/Cb hybridizing sequences as indicated by arrows in Fig 5B and D. We conclude that 12 TγLPD cases displayed rearrangements occurring in the $T_{\gamma}C2$ 5′ flanking sequences involving the J2 region and T_{γ} variable region sequences.

The 5.5-kb *Eco*RI band hybridizing to the Ca/Cb probe showed equal intensity with the control bands in five other cases of TγLPD, which suggested that the $T_{\gamma}C1$ locus was not deleted (see Fig 5, lane 3). Hybridization of *Sst*I-digested DNAs to a Jx2 probe can detect rearrangements in the 5′ flanking regions of the $T_{\gamma}C1$ locus. New *Sst*I restriction fragments hybridizing to the pJx2 probe were detected in three TγLPD cases, which suggested the occurrence of $T_{\gamma}C1$ gene rearrangements (Fig 5C, lane 3). Hybridization of the same blots to a T_{γ} variable region probe (V2) showed that the new bands also contained variable region sequences as indicated by arrows in Fig 5C and D. Finally, in two cases (Table 2), the T_{γ} gene was found in its germline configuration using all of the combinations of restriction enzymes and probes described (see Fig 5A, B, C, and D, lane 4 for a representative case). As in the case of the T_{β} gene, these two cases also lacked the CD3 antigen (Table 2). We conclude that the T_{γ} gene is rearranged in all $CD3^{+}$ cases of TγLPD but it is retained in its germline configuration in the less frequently occurring $CD3^{-}$ cases of TγLPD. In addition, T_{γ} gene rearrangements using $T_{\gamma}C1$ or $T_{\gamma}C2$ are present in $CD3^{+}$ TγLPD cases in similar proportions to those observed in T cell tumors.

Two and one representative cases of $CD3^{+}$ and $CD3^{-}$ TγLPD respectively were also analyzed for T cell receptor gene expression. The two $CD3^{+}$ TγLPD cases displayed expression of full-length mRNAs from T_{β}, T_{α}, and T_{γ}, whereas the $CD3^{-}$ TγLPD case displayed no hybridization signal for all the T cell receptor gene probes (data not shown).

In an effort to elucidate whether the $CD3^{+}$ TγLPD with rearranged and expressed T cell receptor genes produces a "conventional" cell surface α/β heterodimer or the recently described T_{γ}-containing T cell receptor,[43-47] we investigated seven cases of $CD3^{+}$ TγLPD with MoAb WT31, which reacts with a common epitope of the α/β heterodimer but not with T_{α}^{-}, T_{β}^{-}, T_{γ}^{+} cells.[37,43-47] In preliminary experiments we confirmed that $CD3^{+}$, $CD8^{-}$, $CD4^{-}$ clones were $WT31^{-}$.[43-47] As summarized in Table 3, all seven cases were $WT31^{+}$. In case 15, in spite of the monoclonal nature of the $CD3^{+}$ expansion revealed by Southern blot analysis (see earlier), a fraction of $CD3^{+}$ cells were not appreciably stained by the WT31 MoAb. It is of interest in this context that all TγLPD cases were $CD8^{+}$ (as usually observed)[16,42] and that T cell clones expressing a T_{γ}-containing receptor are CD8−, CD4−.[43-45] Thus, the observation of WT31 reactivity together with the finding of T_{α} and T_{β} mRNA and CD8 expression suggests that the majority of TγLPD express a "conventional" α/β heterodimer on their surface.

DISCUSSION

LGL/NK cells constitute a heterogeneous population that cannot be clearly assigned to a specific lymphoid lineage. We sought to clarify the functional and ontogenic relationship between LGL/NK cells and T cells by analyzing the organization and expression of the T cell receptor genes β, α, and γ in unfractionated normal circulating LGL/NK cells, in phenotypically distinct normal LGL/NK cell subsets, and in neoplastic LGL/NK cell proliferations (TγLPD). The normal circulating LGL/NK cells and the phenotypically diverse LGL/NK subsets that we analyzed, including the T cell–like $CD8^{+}$, $CD2^{+}$ subset, displayed the germline configuration and/or lacked expression of the T cell receptor β, α, and γ genes. The lack of T_{β}/T_{α} rearrangement/expression together with the lack of CD3 antigen expression confirms the notion that LGL/NK immune function is clearly distinct from T cell immune function. The lack of T_{γ} gene rearrangement/expression in LGL/NK cells and subsets strongly suggests that LGL/NK cells are ontogenically distinct from T cells because T_{γ} gene rearrangements are believed to represent a very early event during thymic development.[14] Taken together, these data favor the hypothesis that LGL/NK cells represent a cell lineage that is independent and distinct from the T cell lineage. In further support of this conclusion, it has recently been shown that C.B-17 *scid* mice that lack functional T and B cells and whose bone marrow fails to repopulate the thymus have a normal content of NK progenitor cells in the bone marrow.[48]

TγLPD are believed to represent the transformed counterparts of normal LGL/NK cells. Yet, we observed heterogeneity of the TγLPD cases analyzed with respect to their expression of the CD3 antigen and the organization of the T_{β} and T_{γ} loci. The minority of cases (2/17) were $CD3^{-}$ and shared important immunogenotypic, phenotypic, and functional properties with normal circulating LGL/NK cells. They showed germline organization of the T_{β} and T_{γ} genes, an absence of CD3 antigen expression, expression of the Fc_{γ} receptor, and in vitro NK/ADCC activity. Thus, these cases may represent the neoplastic correlate to the major circulating LGL/NK cell population. The remaining 15 cases of TγLPD displayed a combination of the phenotypic, functional, and immunogenotypic properties seen in both T cells and LGL/NK cells but distinct from any single subset of LGL/NK cells that we have analyzed (Tables 1 and 2). Specifically, they all showed T_{β} and T_{γ} gene rearrangements and CD3 antigen expression, typical of mature T cells. They also expressed the Fc_{γ} receptor and exhibited NK and/or ADCC activity, typical of LGL/NK cells.

On the basis of the combined functional, phenotypic, and genotypic analysis of normal and neoplastic T lymphocytes and LGL/NK cells, three distinct populations can be identified: (*a*) major histocompatibility complex (MHC)-restricted T cells and peripheral T cell neoplasms, CD3$^+$, Fc$_\gamma^-$, T$_\beta^+$, T$_\gamma^+$, ADCC$^-$; (*b*) MHC-nonrestricted LGL/NK cells and two of 17 TγLPD, CD3$^-$, Fc$_\gamma^+$, T$_\beta^-$, T$_\gamma^-$, ADCC$^-$; and (*c*) 15 of 17 TγLPD, CD3$^+$, Fc$_\gamma^+$, T$_\beta^+$, T$_\gamma^+$, ADCC$^+$. This raises the question of the relationship of the majority of TγLPD populations, the CD3$^+$ Fc$_\gamma^+$ T$_\gamma$LPD cells, to normal T cells and/or LGL/NK cells. They may represent T cells or LGL/NK cells that have acquired atypical properties during the transformation process or the neoplastic expansion of a separate normal cellular counterpart. Although several authors have reported that Fc$_\gamma$ receptors are not present on the surface of CD3$^+$ T lymphocytes isolated from peripheral blood, concanavalin A–activated cultures, or mixed lymphocyte response cultures,[49-51] the coexistence of Fc$_\gamma$ receptors and CD3 antigen has been reported in selected conditions, eg, the peripheral blood of patients with lymphoproliferative disorders[52,53] or in a variety of in vitro cultured T cell clones (NK-like clones).[54] This functionally unique subset of cytotoxic mononuclear cells, identified by the coexpression of the CD3 antigen and the Fc$_\gamma$ receptor,[55] may be the normal counterpart of the CD3$^+$, Fc$_\gamma^+$ TγLPD. In normal peripheral blood CD3$^+$, Fc$_\gamma^+$ cells represent a minor, usually undetectable ($<1\%$) lymphoid population,[55] which, given its low frequency, was not amenable to analysis by the Southern and Northern blot techniques used in the present study.

The ontogenic relationship between the CD3$^+$, Fc$_\gamma^+$ cell type (TγLPD or NK-like T cells) and T cells or LGL/NK cells bears consideration. Conflicting data have been reported with respect to this issue. Hercend et al reported the isolation of NK-like T cell clones from peripheral T cells,[54] which lent credence to the hypothesis of an ontogenic relationship between T cells and NK-like T cells. However, NK clones bearing rearranged and expressed T cell receptor genes were isolated from the spleens of athymic nude mice by Ikuta et al,[18] which favors the hypothesis of an ontogenic relationship between NK cells and NK-like T cells. In an effort to analyze the relationship between LGL/NK cells and NK-like T cells, in preliminary experiments we have analyzed the in vitro effect of interleukin 2 (IL-2) on purified normal LGL/NK cells. We observed that three to five days after the addition of recombinant IL-2 to the culture the majority of the cells were CD3$^+$, T$_\beta^+$, T$_\alpha^+$, T$_\gamma^+$ and displayed all the functional (NK and ADCC activity) and phenotypic (AB8.28 and HNK-1 antigens) features of LGL/NK cells (unpublished observations). Thus, as suggested by Ikuta et al,[18] CD3$^+$, T$_\beta^+$, Fc$_\gamma^+$ cells may represent a maturative step of LGL/NK cells and may provide a rationale for the ontogenic relationship between the majority of TγLPD and normal LGL/NK cells.

Several recent papers[43-44,56] have described a small population of CD3$^+$, WT31$^-$, CD4$^-$, CD8$^-$ T lymphocytes that have rearranged T$_\gamma$ genes, express T$_\gamma$ protein on the cell surface, and display non–MHC-restricted cytotoxic activity and ADCC. These findings raise the question of the role of the T$_\gamma$ gene product in NK or NK-like cytotoxicity. Our observations that LGLs, which account for virtually all peripheral blood NK activity, maintain the T$_\gamma$ gene in its germline configuration and that CD3$^+$, CD8$^+$, TγLPD with non–MHC-restricted cytotoxicity express T$_\alpha$ and T$_\beta$ mRNAs and display WT31 reactivity suggest that the expression of a T$_\gamma$-containing antigen recognition structure is not an absolute requirement for non–MHC-restricted cytotoxicity.

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Ultrastructural Analysis of Murine Megakaryocyte Maturation In Vitro: Comparison of Big-Cell and Heterogeneous Megakaryocyte Colonies

By Paula E. Stenberg and Jack Levin

Two morphologically distinct types of murine megakaryocyte (MK) colonies are present after three to seven days in soft agar culture: (*a*) "big-cell" colonies composed of ten to 30 large, mature-appearing megakaryocytes and (*b*) "heterogeneous" colonies consisting of approximately 100 or more cells at various stages of differentiation. Cytochemical and immunocytochemical techniques were used to study MK maturation in colonies as well as normal mouse bone marrow. Acetylcholinesterase (AChE), a specific marker for murine platelets and MK, was found in the perinuclear cisterna, endoplasmic reticulum, and occasionally, Golgi cisternae of MK in three-day big-cell colonies and immature bone marrow MK. MK in seven-day big-cell colonies and mature bone marrow MK showed additional reaction sites in the demarcation membrane system and occasional granules. In seven-day heterogeneous colonies, small cells resembled immature bone marrow MK with respect to AChE localization, whereas large cells corresponded to mature bone marrow MK. With immunogold procedures at the ultrastructural level, polyclonal antibodies against human platelet membrane glycoprotein IIIa and antimouse platelet antiserum labeled bone marrow MK and all MK from colonies grown in soft agar cultures for three to seven days. Granulocytes and macrophages in both bone marrow and soft agar cultures were negative for AChE and these immunocytochemical markers. These data indicate that the pattern of expression of AChE during maturation of MK is similar in vivo and in vitro and demonstrate, when using this marker at the fine-structural level, that a greater range of MK maturational stages is present in heterogeneous colonies than is observed in MK in big-cell colonies. Furthermore, we have confirmed that small cells in heterogeneous colonies are MK and that these colonies are composed solely of MK and their precursors.

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TECHNIQUES HAVE EMERGED that permit the identification and study of immature megakaryocytes. One technique is the ability to support proliferation and differentiation of the progeny of megakaryocyte progenitors (megakaryocyte colony–forming cells, Meg-CFC) in vitro, in agar,[1] in methyl cellulose,[2] or in a plasma clot.[3] At least two types of murine megakaryocyte colonies have been recognized in soft agar cultures.[4-8] One type is typically composed of ten to 30 large megakaryocytes that are mature in appearance ("big-cell" type). The other ("heterogeneous" type) is composed of more cells (often >100), some of which are small and immature as evaluated in part by staining for acetylcholinesterase (AChE). In contrast to big-cell colonies, heterogeneous colonies markedly increase in size after their initial appearance.[5,8] Because both types of colonies appear simultaneously in agar cultures, they are probably the progeny of different populations of precursors.[5,8] An important feature of soft agar cultures is that entire, intact megakaryocyte colonies, without contamination by other cell types, may be obtained for the study of megakaryocyte maturation.

The second technique uses markers specific for the megakaryocyte/platelet lineage to identify immature megakaryocytes on a phenotypic basis. AChE staining has proved extremely useful in the identification of cat,[9,10] rat,[9-11] and murine[9,10,12] platelets and megakaryocytes. More recently, antibodies specific for platelet membrane glycoproteins have been used in the identification of human[13-17] and murine[18] megakaryocytes in vivo and in vitro. When immunofluorescence techniques and antibodies directed against platelet glycoproteins were used, both small megakaryocyte precursors and mature megakaryocytes were labeled.[13,15-17,19] At present, however, only limited morphological observations of megakaryocytes in tissue culture systems (particularly those obtained from soft agar culture systems) have been reported.[18,20,21] Specific markers have been used in in vitro systems for studies of the transitional maturational stages between Meg-CFC and megakaryocytes. For example, it has been demonstrated in the mouse that the small AChE-positive cell represents an intermediate stage between Meg-CFC and mature megakaryocytes,[12,22,23] and small cells expressing platelet glycoproteins or cytoplasmic factors have been described in humans both in vitro (in plasma clot cultures) and in vivo.[13,15-17,24] More recently, an immunofluorescence study by Levene et al[25] has suggested expression of platelet glycoprotein IIb/IIIa at the level of the committed megakaryocyte progenitor because an antibody directed against the platelet glycoprotein IIb/IIIa complex inhibited the growth of human megakaryocyte colonies. Another study by Fraser et al[26] has presented evidence that the human pluripotential hematopoietic stem cell expresses platelet lineage–specific markers.

In our previous paper[18] using specific markers at the light microscope level, we observed immunoperoxidase label in most immature cells of megakaryocyte colonies on 2-μm-thick plastic sections. In this investigation, we used specific markers at the fine-structural level to more fully characterize megakaryocytes grown in soft agar cultures because this allows the examination of patterns of protein expression

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in megakaryocytes at early time points in culture (and hence presumably at various stages of differentiation) and, particularly, the study of small, immature cells that partially constitute the heterogeneous megakaryocyte colonies. In contrast to bone marrow cell suspensions, these megakaryocyte colonies permit the study of purified populations of cells of the megakaryocytic lineage at different stages of maturation, without the requirement for initial physical separation from other cell lineages. We specifically compared at the ultrastructural level the pattern of expression of AChE and the distribution of plasma membrane glycoproteins in heterogeneous megakaryocyte colonies and big-cell megakaryocyte colonies to determine whether this system might be useful to study megakaryocyte differentiation.

MATERIALS AND METHODS

Mice

C57BL/6J mice, obtained from Simonsen Laboratories, Inc (Gilroy, CA), were used for these experiments. These mice were bred under specific pathogen-free conditions and then maintained under conventional conditions.

Preparation of Murine Bone Marrow Samples

Mouse femoral marrow samples were obtained from animals killed by cervical dislocation. A 25-gauge needle was inserted into the proximal end of the femur, and fixative was gently pushed through the length of the bone to remove the marrow core. The marrow cells were dispersed into a single-cell suspension by gentle pipetting. The cells were fixed in either 1.44% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 6.8, for 1.5 hours at ambient temperature for AChE staining or in 2% paraformaldehyde with 0.05% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for two hours at ambient temperature for immunocytochemical staining.

Preparation of Murine Megakaryocyte Colonies

Spleen cells were obtained as previously described,[27] and these were used rather than bone marrow because spleen cells produce a greater proportion of megakaryocyte colonies. Cultures of spleen cells were maintained in 0.3% agar in Dulbecco's modified Eagle's medium as described previously.[27] The final concentration of horse serum was 20%. One milliliter of a splenic cell suspension in agar medium was cultured with 0.1 mL of pokeweed mitogen stimulated, spleen cell–conditioned medium and incubated for three to seven days in a humidified atmosphere of 10% CO_2 in air. In this culture system, megakaryocyte colonies achieve their maximum frequency and ploidy (DNA) levels after seven days.[5,27] At various times, colonies were removed from culture dishes with a finely drawn Pasteur pipette and placed either in a vial containing fixative or a small drop of fixative. In the latter case, an equal volume of warm 3% agar was layered onto the colony-fixative drop, allowed to harden, and removed as a disc into a Petri dish of fixative. Fixatives were as described earlier. Granulocyte and macrophage colonies, which also were present in this culture system, were similarly obtained and studied. After fixation, the samples were rinsed in the appropriate wash buffer.

Cytochemical Procedures for Detection of AChE

AChE cytochemistry was performed on suspensions of bone marrow cells or colonies obtained from soft agar cultures by using the technique of Tranum-Jensen and Behnke[22] as modified by Paulus et al.[28] Control preparations included the omission of substrate or the substitution of butyrylthiocholine iodide for acetylthiocholine iodide (Sigma Chemical Co, St Louis). Other samples were preincubated and stained in the presence of 10^{-5} mol/L eserine (Sigma), an inhibitor of both AChE and nonspecific cholinesterase. The samples were then processed for electron microscopy.

Immunocytochemical Procedures

Immunocytochemical procedures on suspensions of bone marrow cells or colonies obtained from soft agar culture were performed as follows. After fixation, the cells were rinsed several times in 0.1 mol/L phosphate buffer, pH 7.4, with 50 mmol/L NH_4Cl and 2% sucrose. They then were incubated with rabbit antihuman glycoprotein IIIa serum (a gift of Dr Rodger P. McEver, San Antonio, TX) or rabbit antimouse platelet antiserum (PAS)[18] (both of which were diluted 1:500 in phosphate-buffered saline) overnight at 4°C. We have previously demonstrated that antisera directed against human platelet antigens identify murine megakaryocytes.[18] Rabbit antimouse PAS was prepared and carefully absorbed with both RBC and WBC as described previously,[27] and an immunoblot assay with solubilized platelet proteins as the target antigens demonstrated that this PAS was active against multiple murine platelet proteins ranging from less than 45 kilodaltons (kD) to more than 300 kD, the resolving limits of the gel system.[29] The most intense labeling occurred against the region of the gel that contained the platelet glycoprotein IIb/IIIa complex. Microscopy with immunoperoxidase–labeled reagents demonstrated no reactivity of the PAS against nonmegakaryocytic bone marrow cells or granulocyte or macrophage colonies,[18] whereas megakaryocytes of all maturational stages were labeled by this polyclonal PAS.[18]

Subsequently, bone marrow cells or colonies were rinsed in phosphate-buffered saline several times and then incubated with protein A–gold probes (5-, 10-, or 15-nm-sized particles) (Janssen Pharmaceuticals, Beerse, Belgium) diluted 1:20 in 20 mmol/L Tris buffer, pH 8.2, with 0.9% NaCl and 0.1% bovine serum albumin. The samples were rinsed four times with the same Tris buffer and then fixed in 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, with 1% sucrose for 45 minutes. They were left overnight at 4°C in 0.1 mol/L cacodylate buffer, pH 7.4, with 1% sucrose and then processed for electron microscopy as will be described. Control procedures included the substitution of irrelevant antibodies, buffer, or preimmune serum for the primary antibodies.

Preparation of Bone Marrow and Colony Samples for Transmission Electron Microscopy

Fixed, stained samples were osmicated (2% OsO_4 in veronal-acetate buffer) and then dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon. Thin sections (100 nm) were cut, stained with either uranyl acetate and lead citrate or lead citrate alone, and examined with a Philips EM-201 transmission electron microscope.

RESULTS

Classification of the maturational stages of megakaryocytes is complex because cytoplasmic and nuclear development are not synchronous events. The present study is primarily based on the system proposed by Paulus[30] in his ultrastructural studies of guinea pig megakaryocytes and used by Bentfeld-Barker and Bainton in their ultrastructural studies of rat and human megakaryocytes.[31,32] In brief, immature megakaryocytes are small cells measuring 6 to 24 µm in the long axis,[33] containing a large, round, indented, or lobed nucleus[23] with prominent nucleoli, and having a high nuclear:cytoplasmic ratio. These cells contain few cytoplasmic organelles other than polyribosomes and mitochondria.

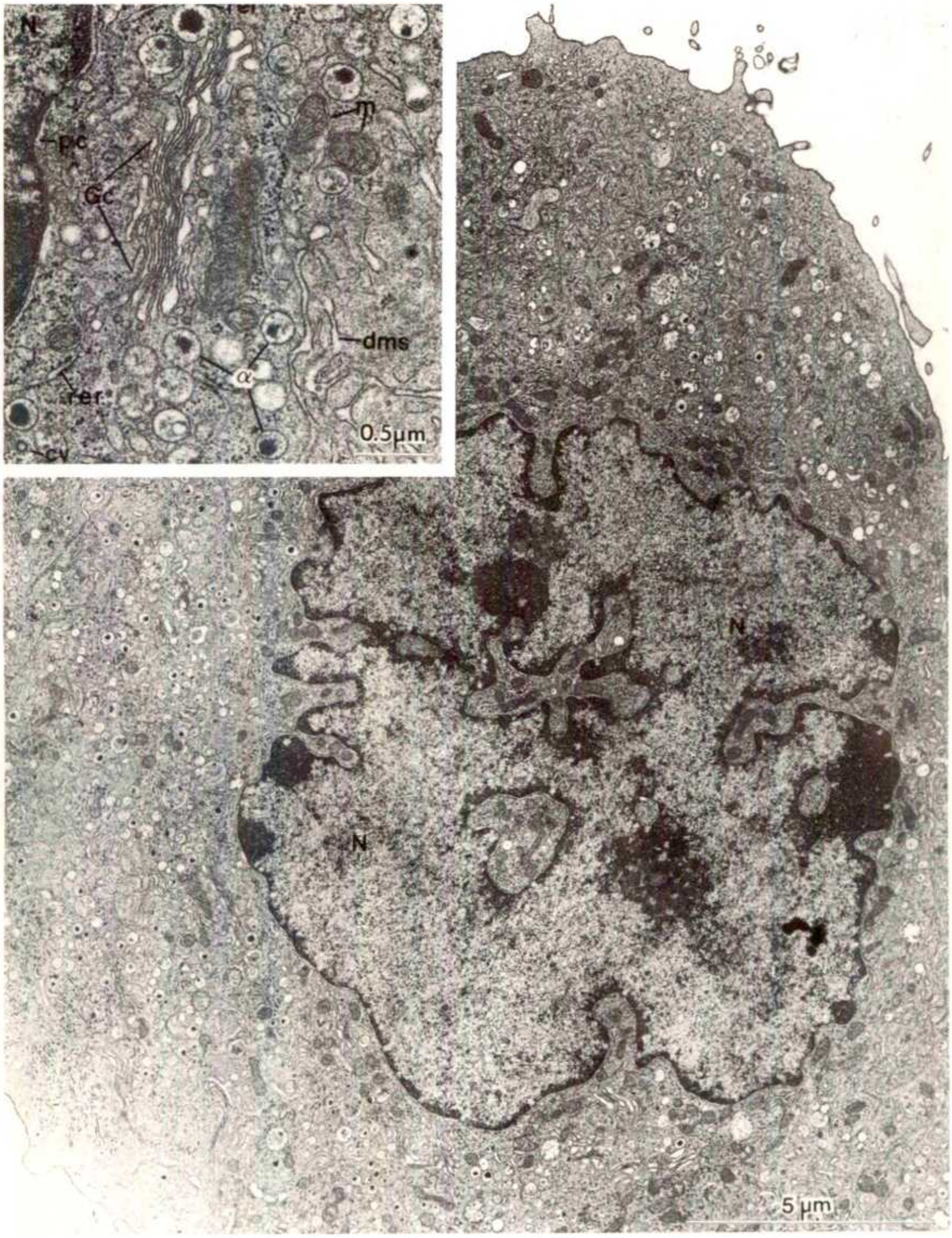

Fig 1. A mature mouse megakaryocyte obtained from a seven-day big-cell megakaryocyte colony. The abundant cytoplasm contains numerous profiles of rough endoplasmic reticulum, demarcation membranes, mitochondria, and α granules, which resembles that of a mature mouse bone marrow megakaryocyte. At higher magnification of the Golgi region of this cell (insert), several cisternae of the Golgi complex (Gc) are evident. Many α granules (α) can be identified in this field. N, nucleus; rer, rough endoplasmic reticulum; dms, demarcation membrane system; cv, coated vesicle; m, mitochondria; pc, perinuclear cisterna (original magnification ×8,000; inset, ×25,000; current magnification ×7,440; inset ×23,250).

Maturing megakaryocytes are heterogeneous in size (14 to 30 μm)[33] and in the level of polyploidy (8 to 32N). They contain a large protein synthetic apparatus and variable numbers of demarcation membranes and α granules. Mature megakaryocytes are approximately 20 to 50 μm in diameter[33] with a low nuclear:cytoplasmic ratio and contain an extensive demarcation membrane system and a reduced protein synthetic apparatus. In contrast to murine bone marrow in which immature megakaryocytes constitute only a small proportion of the cells and are difficult to identify without the use of specific markers, megakaryocyte colonies from soft agar cultures allow the study of sufficient populations of cells of the megakaryocyte lineage that may be more precisely evaluated (ie, in regard to age) than in the bone marrow.

Megakaryocyte colonies present after seven days in soft agar culture (at which time the maximum number of megakaryocyte colonies has been reached) were composed of megakaryocytes at various stages of maturation, from immature to mature cells. Morphologically, mature cells in big-cell and heterogeneous colonies (Fig 1) resembled bone marrow megakaryocytes with the following exceptions. These cells had greater numbers of multivesicular bodies, which presumably contain agar ingested from the surrounding culture medium, than bone marrow megakaryocytes. Additionally, the number of α granules that were uncondensed was greater in megakaryocytes in colonies than in bone marrow megakaryocytes. The size of mature megakaryocytes in seven-day colonies approximated the size of mature bone marrow megakaryocytes, although no evidence of platelet production was observed. At least two thirds of maturing (Fig 2) and mature megakaryocytes examined from both big-cell and heterogeneous colonies contained demarcation membranes only in one area of the cell cytoplasm. These membranes had fewer connections to the megakaryocyte plasma membrane and compartmentalized less cytoplasm than did membranes in bone marrow megakaryocytes. Areas of these demarcation membranes in cells from colonies did not contain many other organelles such as mitochondria and α granules in contrast to bone marrow megakaryocytes, which exhibited a more uniform distribution of organelles. The appearance of these cells partially resembled that of megakaryocytes described previously by Zucker-Franklin and Petursson.[34]

Distribution of AChE in Murine Bone Marrow Megakaryocytes and Megakaryocytes in Soft Agar Culture

Bone marrow megakaryocytes. In immature mouse bone marrow megakaryocytes, reaction product for AChE was confined to the perinuclear cisterna and endoplasmic reticulum (Fig 3A). Few granules and demarcation membrane system profiles were observed in these cells and, if present, were generally unreactive. Occasionally, mouse erythroblasts also demonstrated AChE reaction product in the perinuclear cisterna and endoplasmic reticulum, as reported previously by Paulus et al.[28] In mature mouse bone marrow megakaryocytes, reaction product for AChE was observed in the perinuclear cisterna, endoplasmic reticulum, some cisternae of the Golgi complex, some profiles of the demarcation membrane system, and occasional granules (Fig 3B). All other bone marrow cells including eosinophils were completely unreactive for AChE. Control samples in which specific substrate was either omitted or substituted with butyrylthiocholine or in which preincubation and incubation with eserine was performed resulted in megakaryocytes that were negative for AChE (not shown).

Megakaryocytes in colonies in soft agar culture. Most megakaryocytes in colonies, after three days of soft agar culture, were immature in appearance with a high nuclear:cytoplasmic ratio and few cytoplasmic organelles.

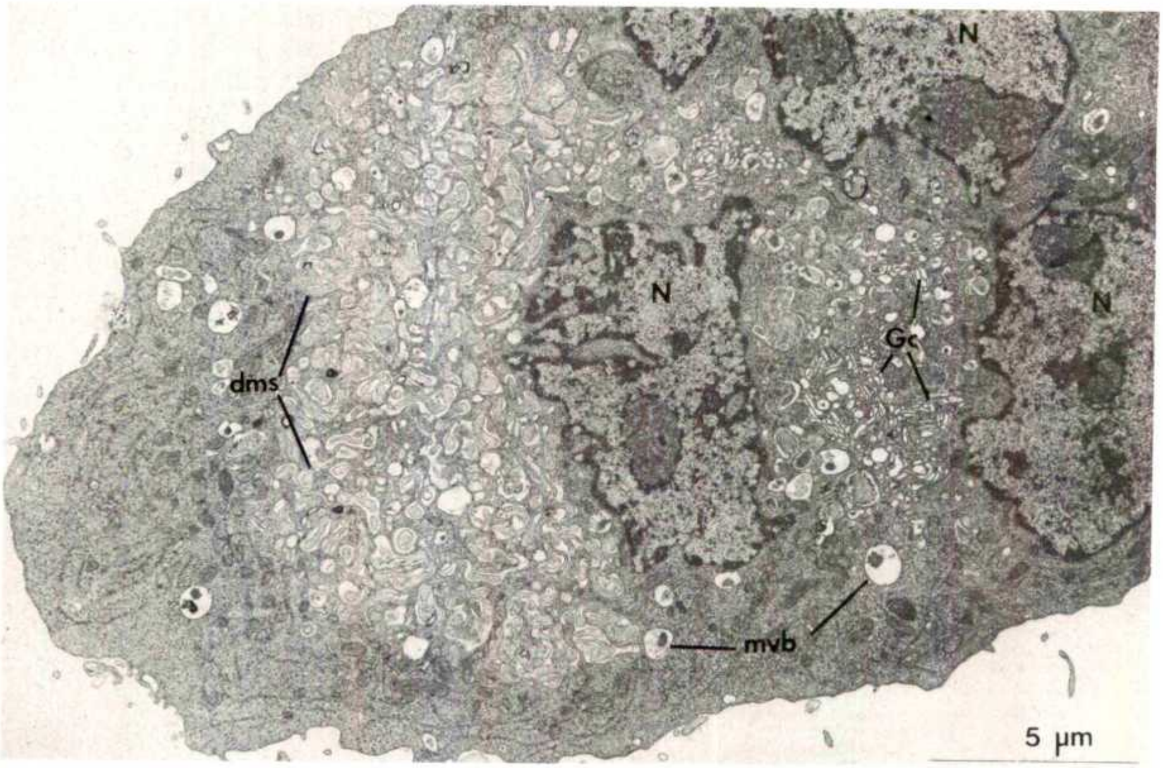

Fig 2. A maturing mouse megakaryocyte obtained from a four-day big-cell megakaryocyte colony. The demarcation membrane system (dms) is concentrated in one area of the cell cytoplasm and contains few other organelles. N, nucleus; Gc, Golgi complex; mvb, multivesicular body (original magnification ×6,000).

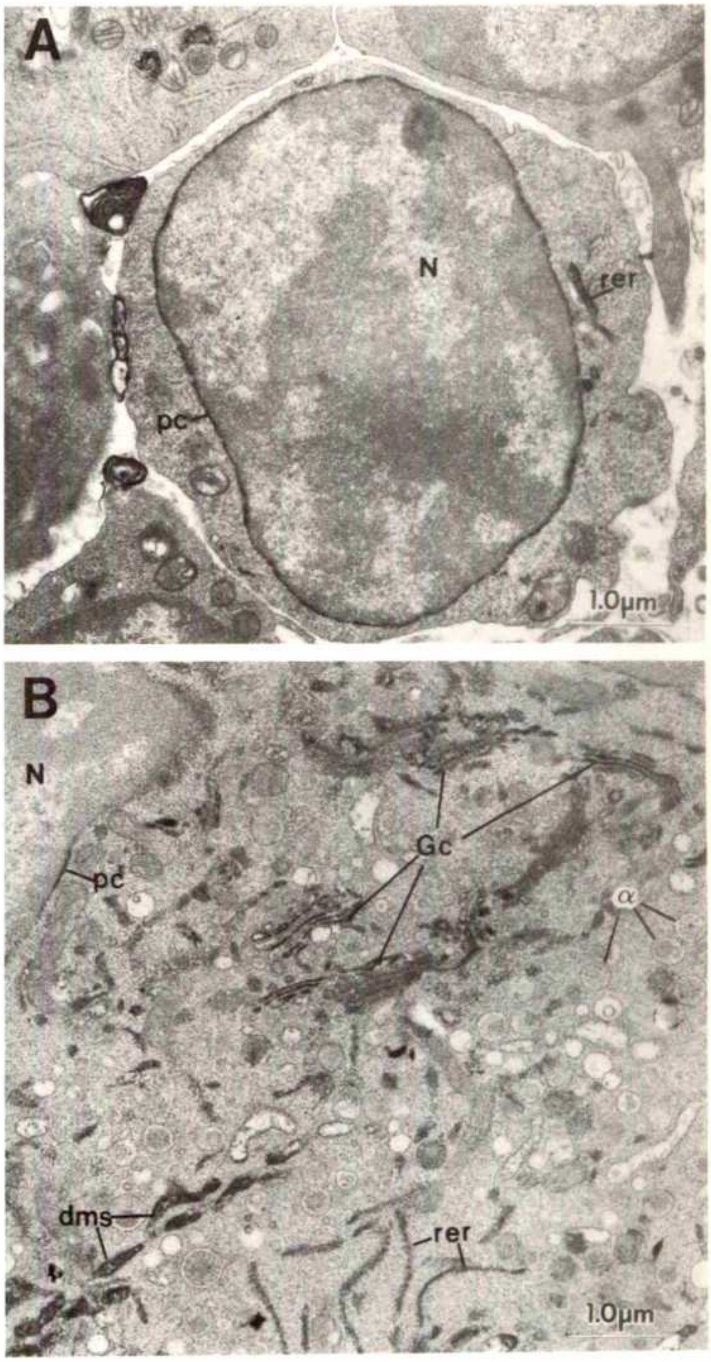

Fig 3. (A) An immature mouse bone marrow megakaryocyte reacted for AChE. Reaction product is confined to the perinuclear cisterna (pc) and rough endoplasmic reticulum (rer). No granules or demarcation membranes are observed (original magnification ×13,500; current magnification ×12,555). (B) A mature mouse bone marrow megakaryocyte reacted for AChE. Reaction product is present in the perinuclear cisterna (pc), rough endoplasmic reticulum (rer), some cisternae of the Golgi complex (Gc), and some profiles of the demarcation membrane system (dms). α Granules (α) are unreactive. N, nucleus (original magnification ×13,500; current magnification ×12,555).

These cells contained reaction product for AChE within the perinuclear cisterna and endoplasmic reticulum (Fig 4A). Occasional megakaryocytes were observed in three-day big-cell colonies, which were considerably more mature and larger in size and contained reaction product in the perinuclear cisterna, many profiles of endoplasmic reticulum, and some cisternae of the Golgi complex (not shown). Megakaryocytes in seven-day big cell colonies resembled mature megakaryocytes in bone marrow with respect to the distribution of AChE. Megakaryocytes in seven-day heterogeneous colonies varied considerably in size and stage of maturation. Small megakaryocytes from these colonies resembled immature megakaryocytes from three day big-cell colonies. All small megakaryocytes examined contained AChE reaction product in the perinuclear cisterna, endoplasmic reticulum (Fig 4B), and some cisternae of the Golgi complex, if this organelle was present. In contrast to data obtained earlier[5] that showed these small cells to be 2N or 4N in ploidy and AChE-negative, we observed positive label in every cell examined at the ultrastructural level. This may be explained by the greater sensitivity of the ultrastructural cytochemical technique as compared with the light microscope level technique. Furthermore, increasing the time of incubation with substrate to 6 hours has demonstrated that all cells in

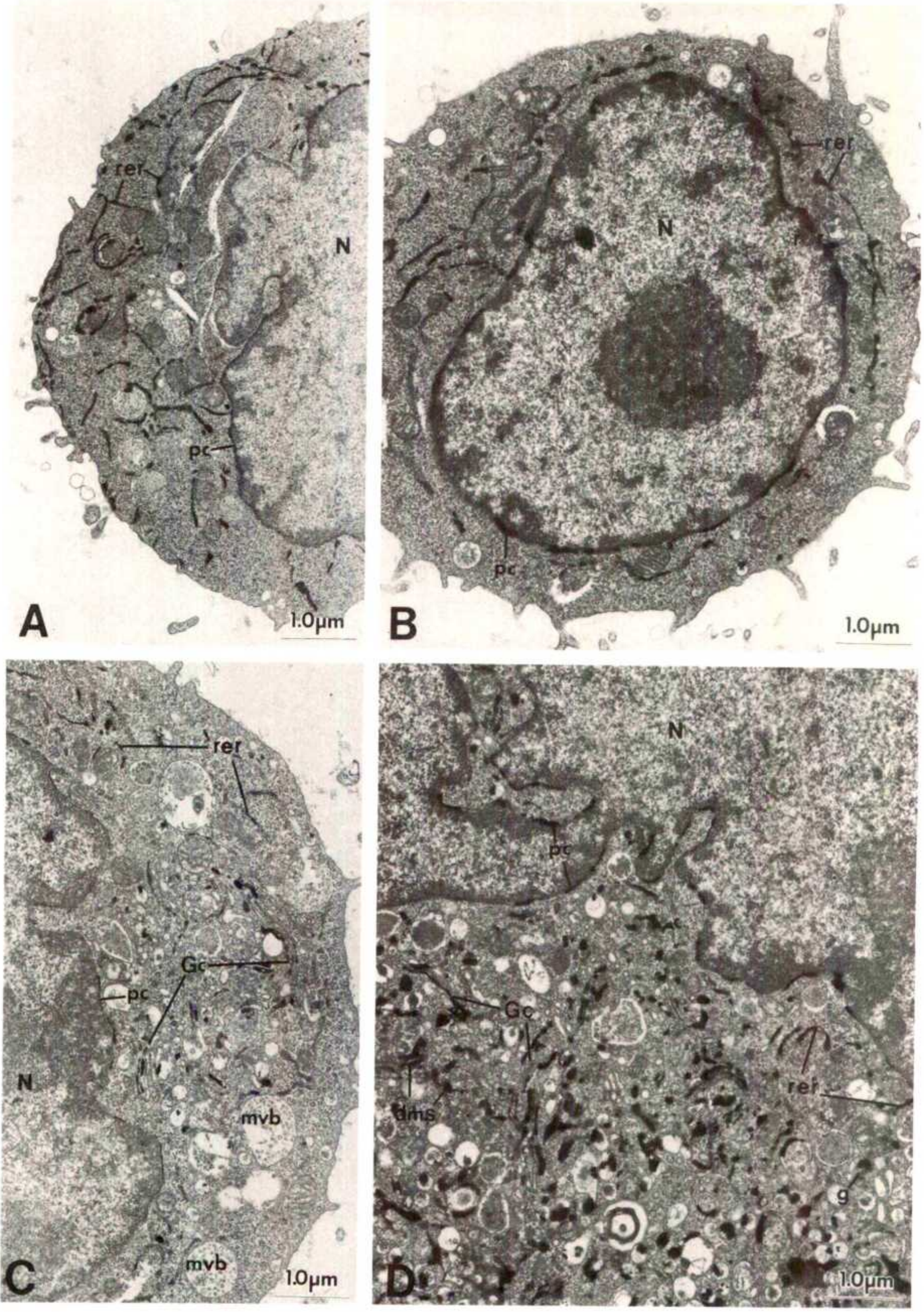

Fig 4. (A) An immature mouse megakaryocyte from a three-day big-cell megakaryocyte colony reacted for AChE. As in Fig 2A, reaction product is confined to the perinuclear cisterna (pc) and rough endoplasmic reticulum (rer) (original magnification ×13,000; current magnification ×12,090). (B) An immature mouse megakaryocyte from a seven-day heterogeneous colony reacted for AChE. As in Figs 2A and 3A, reaction product is found in the perinuclear cisterna (pc) and rough endoplasmic reticulum (rer) (original magnification ×13,000; current magnification ×12,090). (C) A maturing megakaryocyte from a seven-day heterogeneous colony reacted for AChE. Reaction product is present in the perinuclear cisterna (pc), some cisternae of the Golgi complex (Gc), and some profiles of the rough endoplasmic reticulum (rer). Very few demarcation membranes or granules are present in this cell (original magnification ×13,000; current magnification ×12,090); mvb, multivesicular body. (D) A mature megakaryocyte from a seven-day heterogeneous colony reacted for AChE. Reaction product is distributed similarly to mature bone marrow megakaryocytes: perinuclear cisterna (pc), rough endoplasmic reticulum (rer), Golgi complex (Gc), demarcation membrane system (dms), and some granules (g) are labeled. The dms is thicker than the rer. The marked increase in the size of megakaryocytes as they mature in culture is clearly shown by the increased area occupied by the cells in this figure, each of which is shown at the same magnification (original magnification ×13,000; current magnification ×12,090). N, nucleus.

heterogeneous colonies are AChE-positive at the light microscopic level (J. Levin and F. Levin, unpublished observations). Maturing and mature megakaryocytes from seven-day heterogeneous colonies resembled maturing and mature bone marrow megakaryocytes with respect to the distribution of AChE (Figs 4C and 4D). Cells from granulocyte or macrophage colonies grown for three to seven days in soft agar culture were negative for AChE (not shown).

Distribution of Plasma Membrane Glycoproteins in Murine Bone Marrow Megakaryocytes and Megakaryocytes in Soft Agar Culture

Bone marrow megakaryocytes. Mature, recognizable bone marrow megakaryocytes exhibited extensive immunogold label along their plasma membranes when cell suspensions were incubated with either PAS or antiglycoprotein IIIa antibodies and any of the three sizes of protein A–gold probe used, although the greatest amount of label was obtained with PAS followed by protein A–gold (particle size, 5 nm; not shown). All other bone marrow cells were completely negative. Control samples of all bone marrow cells including megakaryocytes in which normal rabbit serum or buffer was substituted for specific primary antibodies also were negative (not shown).

Megakaryocytes in colonies in soft agar culture. All megakaryocytes in either big-cell or heterogeneous colonies after three to seven days in soft agar culture showed immunogold label for PAS or glycoprotein IIIa along the extent of the plasma membrane, regardless of their stage of maturation (Fig 5 and Figs 6A and B). Megakaryocytes in colonies demonstrated a lower concentration of gold particles along plasma membranes than mature bone marrow cells. Although these experiments were technically difficult to perform, all of the immunoreagents passed through the thin agar shell surrounding the colonies.

DISCUSSION

We have investigated the maturation of murine megakaryocytes in soft agar cultures at the ultrastructural level with respect to their morphological appearance and the distribution of AChE and plasma membrane glycoproteins and determined that both big-cell and heterogeneous colonies contain only megakaryocytes. Immature cells in three-day big-cell colonies, seven-day heterogeneous colonies, and bone marrow demonstrated reaction product for AChE predominantly in the perinuclear cisterna and endoplasmic reticulum and also in Golgi cisternae, if present. In contrast, large megakaryocytes from seven-day big-cell and heterogeneous megakaryocyte colonies and mature bone marrow cells were much more reactive than immature cells, with reaction product present in the perinuclear cisterna, endoplasmic reticulum, Golgi complex, demarcation membrane system, and some granules. All cells in megakaryocyte colonies, regardless of the time in culture or extent of morphological maturity, exhibited immunogold label along the plasma membrane with PAS or anti–GP IIIa serum. Importantly, cells in big-cell colonies achieve their ultimate mean ploidy distribution four days after the initiation of cultures, whereas

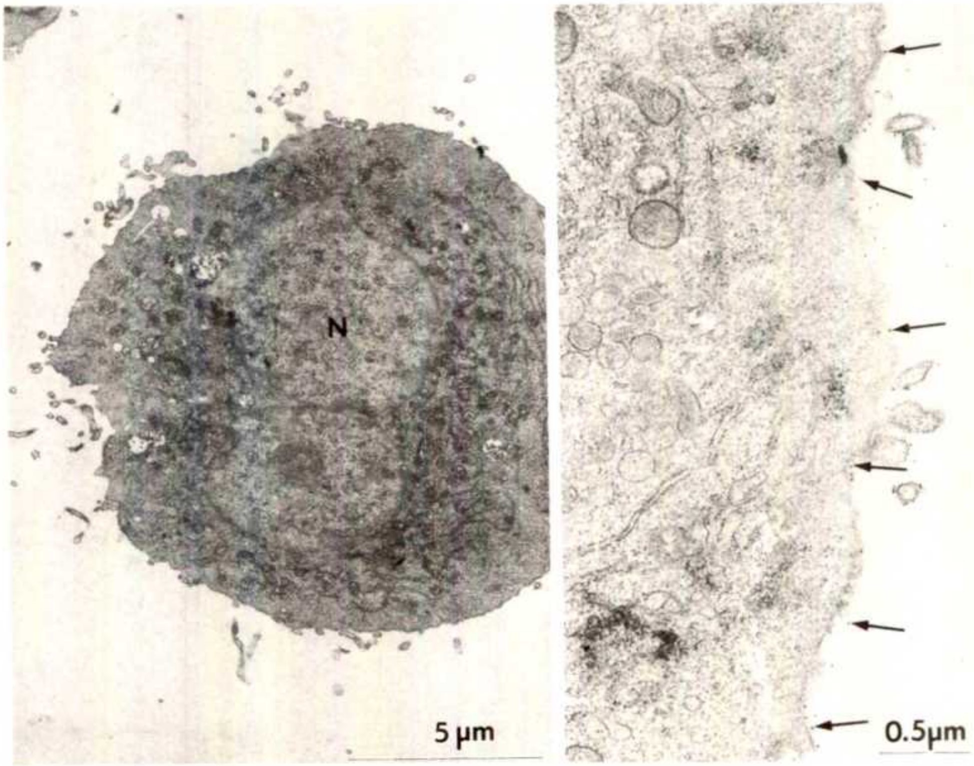

Fig 5. An immature megakaryocyte from a three-day big-cell colony reacted with PAS followed by protein A–gold (size, 10 nm) probe. At higher magnification (inset) the immunogold label is clearly evident along the plasma membrane (arrows). This cell has a high nuclear:cytoplasmic ratio and no dms profiles (original magnification ×5,000; inset ×21,000).

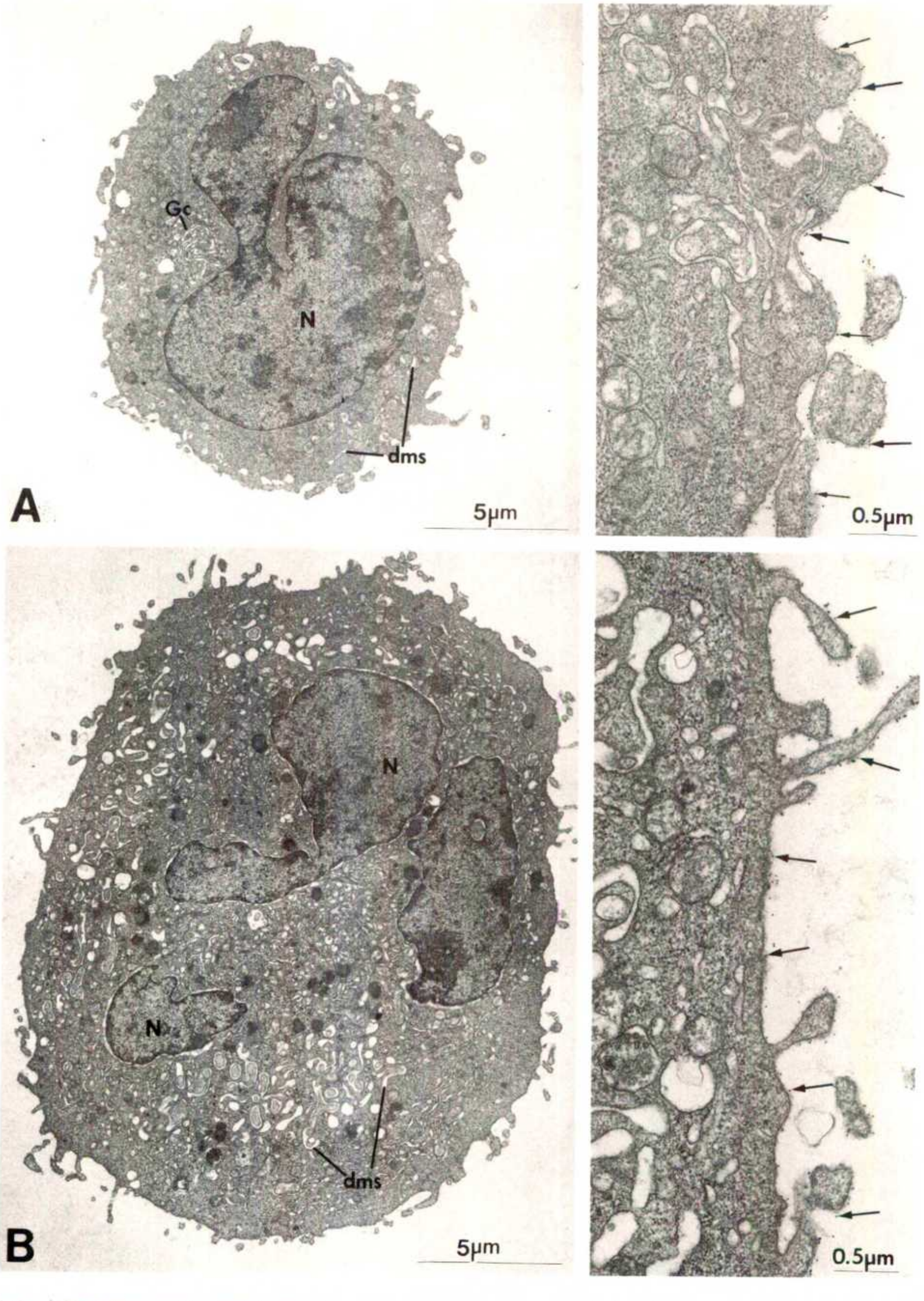

Fig 6. (A) Immature megakaryocyte from a seven-day heterogeneous colony reacted with PAS followed by a protein A–gold (size, 10 nm) probe. At higher magnification (inset) the immunogold label is clearly evident along the plasma membrane (arrows). This cell has a high nuclear:cytoplasmic ratio and only a few dms profiles (original magnification ×5,000; inset ×21,000). (B) Maturing megakaryocyte from a seven-day heterogeneous colony reacted with PAS followed by a protein A–gold (size, 10 nm) probe. Note that this cell is larger, with a lower nuclear:cytoplasmic ratio, and contains more demarcation membranes than the cell in (A). The large, dense granules are multivesicular bodies. At higher magnification (inset) the immunogold label is seen along the plasma membrane at a concentration comparable to that seen in (A) (original magnification ×5,000; inset ×21,000). N, nucleus; dms, demarcation membrane system; Gc, Golgi complex.

the mean ploidy level of heterogeneous colonies continues to increase after four days of culture.[5] Therefore, megakaryocytes express lineage-specific glycoproteins before they attain their final polyploid stage. Pertinently, although heterogeneous colonies are derived from a single precursor cell, they are comprised of megakaryocytes at different stages of maturation (as determined by morphological appearance, size, and AChE localization) in contrast to megakaryocytes in big-cell colonies, which are more uniformly mature in appearance. Because approximately half of the cells in heterogeneous colonies contain 2N or 4N levels of DNA,[5,6] our current fine-structural studies further indicate that cells at low ploidy levels express AChE[35] and lineage-specific membrane glycoproteins.

Previously, staining for AChE has been used to identify immature mouse megakaryocytes.[11,12,23,36] Ultrastructural studies have localized AChE to the protein synthetic apparatus and in a population of granules distinct from α granules in rodent bone marrow megakaryoblasts and megakaryocytes.[10,28] Similarly, we have localized AChE in the endoplasmic reticulum, nuclear envelope, and Golgi complex of immature mouse marrow megakaryocytes and immature megakaryocytes from colonies in soft agar culture. Tranum-Jensen and Behnke also localized AChE to the Golgi complex and profiles of endoplasmic reticulum in small, immature bone marrow cells (6 to 8 μm in diameter), which they considered to be megakaryocyte precursor cells.[22] As previously reported for bone marrow cells,[10,28] the Golgi complex, endoplasmic reticulum, and nuclear envelope as well as demarcation membranes and some granules were labeled in more mature megakaryocytes in soft agar culture. Reaction product in the Golgi complex of cells examined in our study was sometimes confined to only one face of the Golgi complex or was present throughout the Golgi complex, as Tranum-Jensen and Behnke also have previously reported.[10] Therefore, AChE is synthesized and packaged into granules in megakaryocytes maturing in soft agar culture, similarly to megakaryocytes in vivo. This perhaps supports the suggestion of Paulus et al[28] that cholinergic regulation may be involved in the control of megakaryocyte growth and therefore operative both in vivo and in vitro. Additional support for this hypothesis is obtained from the report of Burstein et al[37] that cholinergic agonists increase the frequency of megakaryocyte colonies in vitro.

Platelet peroxidase is currently the earliest recognized marker of human megakaryocytes in vivo and in vitro.[3,15] Vainchenker et al[15] and Vinci et al[14] have determined that the glycoprotein IIb/IIIa complex is also an early antigenic marker of human megakaryocyte maturation because it is present in cells five to six days after the initiation of plasma clot cultures. More recently, Jenkins et al[38] have reported that megakaryocytes derived from human committed megakaryocyte progenitor cells in vitro synthesize glycoproteins IIb and IIIa. Jackson has demonstrated that small rat marrow cells expressing AChE activity also were labeled by fluorescent anti-PAS.[39] We have determined that both AChE and lineage-specific membrane glycoproteins are detectable in immature murine megakaryocytes three days after the culture of Meg-CFC in soft agar, the first day of culture in which murine megakaryocyte colonies can be confidently visually identified under the light microscope. Because it is difficult to reliably retrieve megakaryocyte colonies from soft agar cultures before this time, we were unable to determine which of these markers appears first in our culture system.

It is of interest that during neutrophil differentiation some antigens are lost (as identified by monoclonal antibody MY-10 or those to Ia antigen) or acquired (as identified by monoclonal antibodies Mo1, MY8, 8OH.3, or B34.3) as these cells mature from myeloblasts to polymorphonuclear leukocytes, whereas expression of other antigens (as identified by monoclonal antibodies 82H5, R1B19, and S4-7) occurs throughout the neutrophil maturation sequence.[40] Recently, using monoclonal antibodies with light microscopy, Levene et al[25] have identified three maturational levels in the human megakaryocyte lineage with distinct immunologic phenotypes; some monoclonal antibodies recognize epitopes expressed only at certain stages of maturation (eg, on immature megakaryocytes or on both immature and mature megakaryocytes) or that, in contrast, are present throughout the entire megakaryocyte/platelet lineage. Utilization of the soft agar culture system in conjunction with similar probes to examine murine or human megakaryocyte maturation at the ultrastructural level will yield important information concerning the time of expression and localization of specific proteins during the developmental sequence of this cell. Future studies will examine the effects of administration to mice of PAS[5] or 5-fluorouracil,[41] agents known to perturb thrombopoiesis, to obtain additional information about megakaryocyte differentiation.

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Immunoglobulin and T Cell Receptor β Chain Gene Rearrangement Analysis of Ocular Adnexal Lymphoid Neoplasms: Clinical and Biologic Implications

By Antonino Neri, Frederick A. Jakobiec, Pier-Giuseppe Pelicci, Riccardo Dalla-Favera, and Daniel M. Knowles, II

We investigated the organization of the immunoglobulin heavy chain (IgH) and the T cell receptor beta chain (Tβ) gene loci in 20 ocular adnexal and four extraocular lymphoid neoplasms obtained from 18 patients presenting with an ocular adnexal lymphoid neoplasm. Fifteen ocular adnexal and four extraocular lymphoid neoplasms occurring in 13 patients were classified by morphological examination and immunophenotypic analysis as monoclonal B cell lymphomas. Each one of these 19 lymphoid neoplasms exhibited clonal IgH gene rearrangements upon hybridization of *Eco*RI- or *Hind*III-digested DNA to a heavy-chain joining region (J_H)-specific DNA probe. The bilateral ocular adnexal monoclonal B cell neoplasms occurring simultaneously in two individuals exhibited identical clonal IgH gene rearrangements, which indicated their derivation from an identical B cell clone. The ocular adnexal and the extraocular monoclonal B cell neoplasms occurring in two of three patients also exhibited identical clonal IgH gene rearrangements, which suggested that they too were derived from an identical B cell clone. Five ocular adnexal lymphoid neoplasms were classified by morphological examination and immunophenotypic analysis as benign, polyclonal pseudolymphomas. Three of these five ocular adnexal lymphoid neoplasms exhibited clonal IgH gene rearrangements, which suggested the presence of monoclonal B cell populations that escaped detection by morphological and immunophenotypic examination. None of the 24 pathological samples exhibited clonal Tβ gene rearrangements upon hybridization of *Eco*RI- or *Bam*HI-digested DNA to a Tβ gene DNA probe. The results of these studies demonstrate the value of Southern blot hybridization analysis for clonal IgH and Tβ gene rearrangements in the diagnosis, classification, and investigation of extranodal lymphoid neoplasms originating and/or presenting in the ocular adnexa.

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EXTRANODAL LYMPHOID INFILTRATES, ie, those that originate and/or present outside of the major lymphoid tissue–bearing sites, often represent a difficult clinical and pathological diagnostic dilemma.[1] This is particularly true for small lymphocytic infiltrates, which constitute the majority of the ocular adnexal and pulmonary lymphoid neoplasms and which also occur in the gastrointestinal tract, skin, salivary glands, and other sites.[1,2] The histopathologic criteria commonly used to distinguish benign and malignant extranodal small lymphocytic infiltrates are often incapable of reliably making this distinction,[3] sometimes resulting in inaccurate patient prognostication. Cell marker analyses have shown that extranodal small lymphocytic infiltrates are largely divisable into monoclonal B cell, presumably malignant, small (well-differentiated) lymphocytic lymphomas and polyclonal, presumably benign, pseudolymphomas.[4,5] However, a recent prospective study suggested that disseminated malignant lymphoma occurs with equal frequency in patients with ocular adnexal lymphoid neoplasms belonging to each category.[2] This finding suggests that immunophenotypic analysis also may be incapable of reliably prognosticating patients with extranodal small lymphocytic infiltrates. Moreover, clinical, morphological, and immunophenotypic approaches have not provided significant insight into the pathogenetic relationship between extranodal pseudolymphoma and malignant lymphoma, between extranodal and nodal lymphoid neoplasms, and among multiple extranodal lymphoid infiltrates occurring in the same individual.

The antigen recognition molecules of B and T cells, immunoglobulin (Ig) and T cell receptor(s), respectively, are encoded by genetic loci that undergo somatic recombinations (rearrangements) to become functionally active in mature lymphocytes.[6-8] We and other investigators have demonstrated that clonal rearrangements of the Ig and the T cell receptor β chain (Tβ) gene loci are accurate and objective molecular genetic markers of the lineage and clonality of B and T cells, respectively.[9-13] The demonstration of clonal Ig and Tβ gene rearrangements by Southern blot hybridization analysis has determined the lineage and the clonality of a variety of lymphoproliferative disorders and has become an important adjunct in the diagnosis, classification, and investigation of lymphoid neoplasias.[14-17] However, immunogenotypic analysis has not been systematically applied to an examination of extranodal small lymphocytic infiltrates.

We investigated the organization of the Ig and the Tβ gene loci in 24 morphologically and immunophenotypically well characterized samples of ocular adnexal, eg, conjunctival, lid, orbital, and extraocular lymphoid neoplasms obtained from 18 patients presenting with an ocular adnexal lymphoid neoplasm. This survey included patients with a solitary, unilateral ocular adnexal lymphoid neoplasm without sys-

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Table 1. Clinical Findings in 18 Patients Presenting With Ocular Adnexal Lymphoid Neoplasms

Patient	Age	Sex	Pertinent Prior History	Presentation and Findings	Histopathology	Clonality	Treatment	Clinical Course	Eventual Outcome
1	62	M	36 mo earlier RUL pseudolymphoma; systemic evaluation negative; no therapy	Proptosis, firm nodular RUL mass; systemic evaluation negative	RLH	Polyclonal	Incisional Bx; local irradiation	Asymptomatic	NED: 29 mo
2	55	F	None	7-mo Hx of painless, enlarging LUL; ptosis on LUL, firm mass adherent to orbit; systemic evaluation negative	RLH	Polyclonal	Excisional Bx only	Asymptomatic	43 mo: RUL lymphoid tumor; Bx: DPDL, monoclonal B; local irradiation
3	64	F	None	6-mo Hx of LLL swelling with protrusion of left eye; palpable mass on lower orbital rim with slight proptosis and upward displacement of globe; systemic evaluation negative	RLH	Polyclonal	Subtotal excision only	Asymptomatic	NED: 56 mo
4	51	F	Undocumented Hx of "benign lymphoma" of lung treated by irradiation	36-mo Hx of LLL fullness and 6-mo Hx of LLL swelling; palpable firm LUL fullness; systemic evaluation negative	RLH	Polyclonal	Excisional Bx only	Asymptomatic	NED: 43 mo
5	59	F	None	30-mo Hx of LUL swelling, recent ptosis; CAT scan: left orbital mass; systemic evaluation negative	RLH	Polyclonal	Excisional Bx; local irradiation	Asymptomatic	NED: 44 mo
6	54	M	None	72-mo Hx of right eye prominence; proptosis; systemic evaluation negative	SL	Monoclonal B	Excisional Bx; local irradiation	Asymptomatic	NED: 12 mo
7	64	F	None	4-mo Hx of left ocular proptosis; Firm, rubbery mass on LLL; systemic evaluation negative	IL	Monoclonal B	Excisional Bx; local irradiation	Asymptomatic	NED: 39 mo
8	63	F	Kaposi's sarcoma of lower extremities	2-mo Hx of LUL swelling; firm, nontender, mobile LUL mass; systemic evaluation negative	SL	Monoclonal B	Excisional Bx; local irradiation	Kaposi's sarcoma lesions increase in number	Dead: 46 mo, metastatic lung carcinoma
9	43	M	None	1-mo Hx of RUL ptosis; palpable mass on RUL, RUL ptosis; systemic evaluation negative	DLNC	Monoclonal B	Excisional Bx; systemic chemotherapy for 6 mo	Asymptomatic	NED: 28 mo
10	81	M	None	12-mo Hx of right superior conjunctival mass with occasional blurred vision; firm, salmon-colored lesion on right epibulbar surface extending into superolateral fornix; systemic evaluation negative	SL	Monoclonal B	Excisional Bx; local irradiation	Asymptomatic	NED: 36 mo

11	81	M	2-yr Hx of generalized lymphadenopathy; Bx diagnosis: DPDL	2-mo Hx of painless RUL swelling; firm, salmon-colored mass of superotemporal quadrant; CAT scan: extensive abdominal lymphadenopathy	DLNC	Monoclonal B	Incisional Bx; local irradiation; systemic chemotherapy	Systemic dissemination	Dead: 16 mo, disseminated lymphoma
12	56	F	None	4-mo Hx of RUL mass; firm, rubbery, mobile RUL mass; refused systemic evaluation	SL	Monoclonal B	Excisional Bx; refused further therapy	12 mo: asymptomatic LUL mass; Bx: lymphoma; serum IgMκ spike, refused therapy; 18-mo CAT scan: gastric mass; Bx: lymphoma; systemic chemotherapy	Alive with systemic disease: 26 mo
13	64	F	None	2-mo Hx of RUL mass; firm RUL mass; CAT scans: generalized lymphadenopathy, hepatosplenomegaly	SL	Monoclonal B	Excisional Bx; systemic chemotherapy	Asymptomatic	NED: 14 mo
14	63	M	None	6-mo Hx of LUL ptosis; LUL fullness with ptosis; generalized lymphadenopathy, hepatosplenomegaly, IgGλ monoclonal gammopathy; LN Bx: lymphoma	FLC	Monoclonal B	Incisional Bx; local irradiation; systemic chemotherapy	17 mo: generalized lymphadenopathy; systemic chemotherapy	Dead: 29 mo, disseminated lymphoma
15	51	F	None	18-mo Hx of left eye prominence; palpable mass above orbital rim, beneath LLL; CAT scan: mediastinal and retroperitoneal lymphadenopathy	SL	Monoclonal B	Incisional Bx; systemic chemotherapy	CAT scan: decreased lymphadenopathy	Alive with systemic disease: 12 mo
16	67	F	None	4-mo Hx of LLL mass; firm, nontender LLL mass; generalized lymphadenopathy	SL	Monoclonal B	Excisional Bx; local irradiation	CAT scan: abdominal lymphadenopathy; Bx: lymphoma; LN Bx: lymphoma	Dead: 15 mo, disseminated lymphoma
17	70	F	None	4-mo Hx of bilateral LLL and RUL swellings; firm LLL mass and visible RUL mass with globe displacement	IL IL	Monoclonal B	Bilateral excisional Bx; bilateral local irradiation	CAT scan: retroperitoneal lymphadenopathy; LN Bx: lymphoma; BM Bx: lymphoma; systemic chemotherapy	Alive with systemic disease: 24 mo
18	86	F	None	12-mo Hx of RUL swelling and ptosis, LUL and LLL lumps; palpable RUL, LUL, LLL masses with RUL ptosis; bilateral cervical, axillary lymphadenopathy; LN Bx: lymphoma; BM Bx: negative	F + DSC F + DSC	Monoclonal B	Incisional Bx; local irradiation	Asymptomatic	Alive with systemic disease: 24 mo

Abbreviations: RUL, right upper lid; RLL, right lower lid; LUL, left upper lid; LLL, left lower lid; RLH, reactive lymphoid hyperplasia; SL, small lymphocytic lymphoma; IL, intermediate lymphocytic lymphoma; FLC, follicular large cell lymphoma; DLNC, diffuse large, noncleaved cell lymphoma; F + DSC, follicular and diffuse small cleaved cell lymphoma; DPDL, diffuse poorly differentiated lymphocytic lymphoma; Hx, history; Bx, biopsy; LN, lymph node; BM, bone marrow; NED, no evidence of disease; CAT, computed axial tomography.

temic lymphoma, patients with simultaneous bilateral ocular lymphoid neoplasms, and patients who were discovered to have concurrent ocular adnexal and nonocular lymphoid neoplasms at the time of presentation. The results of these studies demonstrate the utility of immunogenotypic analysis in the diagnosis, classification, and investigation of extranodal lymphoid neoplasms.

MATERIALS AND METHODS

Patients. Eighteen patients presenting with an ocular adnexal lymphoid neoplasm were included in this study (Table 1). These patients were randomly selected from among cases of ocular adnexal lymphoid neoplasia that had been previously well characterized and in which adequate numbers of cells were available for Southern blot hybridization analysis.

Specimens. One sample of heparinized peripheral venous blood and representative portions of the surgical biopsy specimens of 20 ocular adnexa and three lymph nodes, all suspected of involvement by malignant lymphoma, were obtained from these 18 patients. These specimens were collected under sterile conditions during the course of standard diagnostic procedures. A mononuclear cell suspension of greater than 95% viability and free of erythrocytes, dead cells, and cellular debris was prepared from each sample by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation.[18] Immunophenotypic analysis was performed at the time of mononuclear cell isolation before cryopreservation. Excess cells were cryopreserved in vapor-phase liquid nitrogen at −170°C in the presence of dimethylsulfoxide and fetal calf serum.

Histopathology. Hematoxylin-eosin–stained sections were prepared from the paraffin blocks from each biopsy specimen corresponding to the tissue investigated by immunophenotypic and molecular genetic analysis. Each ocular lymphoid neoplasm was classified as an inflammatory pseudotumor, reactive lymphoid hyperplasia, or malignant lymphoma according to previously established histopathologic criteria.[19] The malignant lymphomas were subclassified according to the working formulation of the non-Hodgkin's lymphomas.[20] All additional relevant biopsy specimens also were reviewed, even though these tissues might not have been subjected to special studies.

Immunophenotypic analysis. The proportion of cells in each pathological sample that formed sheep erythrocyte rosettes, expressed surface Ig (sIg) and/or a variety of B- and T cell–associated differentiation antigens, Ia, B1, B4, Leu-14, T1, T3, T4, and T8,[21] was determined in suspensions of viable mononuclear cells by using previously described techniques.[22]

Molecular genetic analysis. DNA was extracted from each of the 24 pathological samples by standard techniques.[23] Twenty micrograms of DNA was digested with the appropriate restriction endonuclease and analyzed for Ig and Tβ gene rearrangements by Southern blot hybridization.[24] Hybridization was performed in 50% formamide, 3× standard saline citrate buffer (SSC) at 37°C for 16 hours. The filters were washed in 0.2 × SSC and 0.5% sodium dodecyl sulfate, pH 7.0, at 60°C for two hours.[25] DNA clones representative of different portions of the Ig and Tβ gene loci were ^{32}P labeled by nick-translation[26] for use as probes. The range of specific activities obtained with this DNA-labeling technique is 2 to 5×10^8 dpm/μg. We have determined, by DNA dilution experiments, that clonal populations representing 5% of the total DNA may be detected by using nick-translated probes and these hybridization and washing conditions. The IgH gene locus was studied by hybridization of *Eco*RI- and *Hind*III-digested DNA to a J region (J_H)-specific probe, a *Bam*HI-*Hind*III 6-kilobase fragment representative of the entire J_H region (kindly provided by Dr Stanley Korsmeyer, National Institutes of Health).[27,28] The Tβ gene locus was analyzed by hybridization of *Eco*RI- and *Bam*HI-digested DNA to a Tβ gene probe that hybridizes to both constant region alleles (a gift from Dr Tak Mak, Ontario Cancer Institute).[11,29]

RESULTS

Clinical features. The age, sex, pertinent prior history, clinical presentation and findings, histopathologic and immunophenotypic diagnosis, form of therapeutic intervention, subsequent clinical course, and eventual outcome of each of the 18 patients included in this study is summarized in Table 1. The patients ranged from 43 to 86 years of age (mean age, 63 years). Six patients were male, and 12 patients were female. Sixteen patients (Table 1, case nos. 1 to 16) presented with unilateral, and two patients (Table 1, cases 17 and 18) presented with simultaneous bilateral, ocular adnexal lymphoid neoplasms. Both patients with bilateral and five of the 16 patients (Table 1, case nos. 11 to 15) with unilateral, ocular adnexal lymphoid neoplasms were discovered to have disseminated systemic lymphoma at the time of presentation. One of these five patients (Table 1, case no. 11) had a history of generalized lymphadenopathy and carried a prior lymph node biopsy diagnosis of "diffuse, poorly differentiated lymphocytic lymphoma." The remaining 11 patients had no evidence of systemic lymphoma based upon radiological and, in some instances, bone marrow examination at the time of presentation. However, patient no. 1 had undergone an excisional biopsy of a histopathologically benign right upper lid mass 3 years earlier. Systemic evaluation was negative at that time, and therapy had never been initiated. Patient no. 4 had an undocumented history of a "benign lung lymphoma" that had been treated by irradiation.

Immunophenotypic and histopathologic analysis. We performed immunophenotypic analysis on each of the 20 ocular adnexal lymphoid neoplasms occurring in these 18 patients and also on three samples of lymph node and one sample of peripheral blood obtained from three of these 18 patients (Tables 2 and 3). The results of these analyses determined that 15 ocular adnexal lymphoid neoplasms occurring in 13 patients (Table 1, case nos. 6 to 18) were monoclonal B cell proliferations. This includes six patients with unilateral ocular lymphoid neoplasms limited to the ocular adnexa, the two patients with simultaneous bilateral ocular adnexal lymphoid neoplasms, and all five patients discovered to have nonocular lymphoid neoplasia. The unila-

Table 2. Results of Immunophenotypic and Molecular Genetic Analysis of Five Ocular Adnexal Pseudolymphomas

	Immunophenotype								Genotype	
Patient	Ia	sIg	κ	λ	T1	T3	T4	T8	IgH	Tβ
1	42	23	13	10	67	69	60	7	G	G
2	36	ND	12	22	57	50	41	13	G	G
3	48	ND	28	18	51	52	49	8	R	G
4	51	ND	12	14	51	55	43	10	R	G
5	53	37	15	16	44	ND	ND	ND	R	G

Numbers indicate the percentage of cells expressing the particular cell marker.

Abbreviations: G, germline; R, rearranged; ND, not done.

Table 3. Results of Immunophenotypic and Molecular Genetic Analysis of 15 Ocular Adnexal and Extraocular Non-Hodgkin's Lymphomas

		Immunophenotype									Genotype	
Patient	Specimen	Ia	B1	sIg	Isotype	T1	E	T3	T4	T8	IgH	$T\beta$
6	Ocular	64	ND	66	MDκ	90	29	30	24	5	R	G
7	Ocular	75	ND	65	Mλ	68	27	21	19	5	R	G
8	Ocular	70	87	75	Mκ	95	11	ND	ND	ND	R	G
9	Ocular	61	ND	59	Gλ	29	38	26	13	17	R	G
10	Ocular	62	ND	84	κ	10	15	11	13	1	R	G
11	Ocular	95	ND	95	λ	95	1	6	2	2	R	G
12	Ocular	90	75	75	MDκ	ND	23	15	10	3	R	G
13	Ocular	75	74	72	MDκ	26	ND	26	23	7	R	G
14	Ocular	69	72	70	Mκ	30	ND	35	18	13	R	G
	Lymph Node	62	65	50	Mκ	36	39	37	18	15	R	G
	Peripheral blood	59	60	60	Mκ	39	33	36	18	9	R	G
15	Ocular	77	83	80	Mκ	13	ND	13	8	2	R	G
	Lymph node	92	90	88	Mκ	8	7	6	4	3	R	G
16	Ocular	95	90	95	MDλ	95	1	ND	ND	ND	R	G
	Lymph node	90	90	95	MDλ	95	3	ND	ND	ND	R	G
17	Ocular-right	85	90	90	MDκ	6	11	5	4	3	R	G
	Ocular-left	66	83	94	MDκ	7	9	6	4	2	R	G
18	Ocular-right	74	81	71	MDκ	18	16	31	21	6	R	G
	Ocular-left	80	84	73	MDκ	19	20	17	12	6	R	G

Numbers indicate the percentage of cells expressing the particular cell marker.

Abbreviations: G, germline; R, rearranged; ND, not done.

teral ocular adnexal lymphoid neoplasms occurring in the five remaining patients (Table 1, case nos 1 to 5) were polyclonal lymphoid proliferations.

Each of the 15 ocular adnexal monoclonal B cell neoplasms contained $\geq$60% B cells expressing either κ or λ light chains. The immunoglobulin isotype was IgMDκ in 7; IgMκ in three; and IgMDλ, IgMλ, and IgGλ in one case each, respectively. We did not determine the Ig heavy chain class in two cases; one of these cases expressed λ and 1 case expressed κ light chains. The bilateral ocular adnexal lymphoid neoplasms occurring in a single individual expressed an identical Ig isotype. Each nonocular lymphoid neoplasm examined expressed the same Ig isotype as the ocular adnexal monoclonal B cell neoplasm occurring in the same patient.

We classified each of the 15 ocular adnexal monoclonal B cell neoplasms as a malignant lymphoma on the basis of morphological criteria. We subclassified these 15 malignant lymphomas as small lymphocytic,[7] intermediate lymphocytic,[3] diffuse large noncleaved cell,[2] follicular and diffuse small cleaved cell,[2] and follicular large cell.[1] The bilateral ocular adnexal lymphoid neoplasms occurring in a single individual were identical morphologically. In addition, each nonocular lymphoid neoplasm was similar morphologically to the ocular adnexal lymphoid neoplasm occurring in the same patient.

Each of the five ocular adnexal polyclonal lymphoid neoplasms contained an admixture of T and B cells. The proportion of T cells ranged between 44% and 69% on the basis of T1 and/or T3 antigen expression. The T cell population consisted of T4$^+$ and T8$^+$ cells in ratios ranging from 3.2 to 8.6. The B cell population consisted of κ and λ light chain–positive B cells in ratios ranging from 0.5 to 1.6. We interpreted these five ocular adnexal lymphoid neoplasms as reactive lymphoid hyperplasias on the basis of morphologic criteria.

Molecular genetic analysis. We investigated the organization of the IgH and the $T\beta$ gene loci in each of the 20 ocular adnexal lymphoid neoplasms occurring in these 18 patients. Each of the 15 ocular adnexal lymphoid neoplasms that we determined to be a monoclonal B cell proliferation by immunophenotypic analysis exhibited clonal IgH gene rearrangements upon hybridization of *Eco*RI- or *Hind*III-digested DNA to a J_H-specific DNA probe. In addition, the ocular adnexal monoclonal B cell neoplasm occurring in each individual exhibited a unique IgH gene rearrangement pattern consistent with a derivation from a distinctive B cell clone (representative data are illustrated in Fig 1). The bilateral ocular adnexal lymphoid neoplasms occurring simultaneously in each of two individuals (Table 1, case nos. 17 and 18) exhibited identical clonal IgH gene rearrangement patterns (Fig 2), which suggested that they are derived from the identical B cell clone and do not represent separate primary neoplasms.[30] None of these monoclonal B cell neoplasms exhibited clonal $T\beta$ gene rearrangements upon hybridization of *Eco*RI- or *Bam*HI-digested DNA to a $T\beta$ gene DNA probe (data not shown). We had previously demonstrated that approximately 11% of lymphoid neoplasms exhibit both clonal IgH and $T\beta$ gene rearrangements, ie, they are bigenotypic.[28] The absence of bigenotypic neoplasms in this series suggests that extranodal lymphoid neoplasms may be bigenotypic less often than are nodal lymphoid neoplasms.

We also examined one peripheral blood and three lymph node samples obtained from three of the patients discovered to have concurrent ocular adnexal and nonocular lymphoid neoplasia (Table 1, case nos. 14 to 16). We demonstrated by immunophenotypic analysis that each of these samples con-

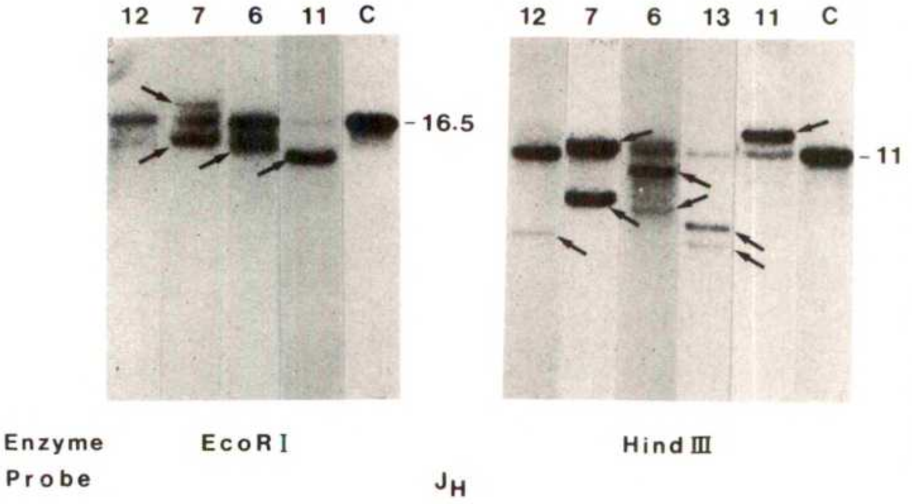

Fig 1. Southern blot hybridization analysis of IgH gene in DNAs extracted from ocular adnexal monoclonal B cell neoplasms. Lanes C contain human fibroblast DNA. The number above each of the other lanes corresponds to the case number in Table 1. The DNAs were digested with *Eco*RI and *Hind*III and hybridized to a joining-region, heavy-chain (J_H) probe. Rearrangement bands are indicated by arrows. Lanes C display the IgH gene germline configuration without rearrangement bands. Each of the 15 ocular adnexal lymphoid neoplasms classified morphologically as a malignant lymphoma and determined to be monoclonal B cell by immunophenotypic analysis exhibited clonal IgH gene rearrangement bands.

tained a monoclonal B cell proliferation expressing the same immunoglobulin isotype as the B cells constituting the patient's ocular adnexal lymphoid neoplasm. Molecular genetic analysis with two different restriction endonucleases, *Eco*RI and *Hind*III, demonstrated that the nonocular lymphoid neoplasms in patients no. 14 and no. 16 exhibited a pattern of clonal IgH gene rearrangement bands that was identical to that expressed by the ocular adnexal lymphoid neoplasms occurring in the same patients (Fig 3). Thus, the ocular adnexal and the nonocular lymphoid neoplasms occurring in these two patients appear to be derived from the same B cell clone and do not represent separate primary lymphoid neoplasms. However, the lymph node obtained from patient no. 15 exhibited a clonal IgH gene rearrangement pattern that was distinct from that of the patient's ocular adnexal lymphoid neoplasm (Fig 3). This case may represent a biclonal B cell lymphoma.[31] None of these nonocular lymphoid neoplasms exhibited clonal Tβ gene rearrangements upon hybridization of *Eco*RI- or *Bam*HI-digested DNA to a Tβ-specific DNA probe.

We had interpreted five unilateral ocular adnexal lymphoid neoplasms (Table 1, case nos. 1 to 5) to be benign, polyclonal pseudolymphomas based upon histopathologic examination and immunophenotypic analysis. None of these five pathological samples exhibited clonal Tβ gene rearrangements upon hybridization of *Eco*RI- or *Bam*HI-digested DNA to a Tβ-specific DNA probe (data not shown). However, two of these five pathological samples (Table 1, case nos. 4 and 5) exhibited clear and easily recognizable clonal IgH gene rearrangement bands upon hybridization of *Eco*RI- or *Hind*III-digested DNAs to a J_H-specific probe (Fig 4). A third sample exhibited a faint nongermline band upon analysis of *Eco*RI-digested DNA and three faint, barely perceptible, IgH gene rearrangement bands upon analysis of *Hind*III-digested DNA, which suggested B cell oligoclonality (Fig 4). The two remaining pathological samples (Table 1, case nos. 1 and 2) exhibited the germline IgH DNA configuration (Fig 4). Thus, three of the five ocular adnexal pseudolymphomas that we investigated contained either oligoclonal or conspicuous mono-

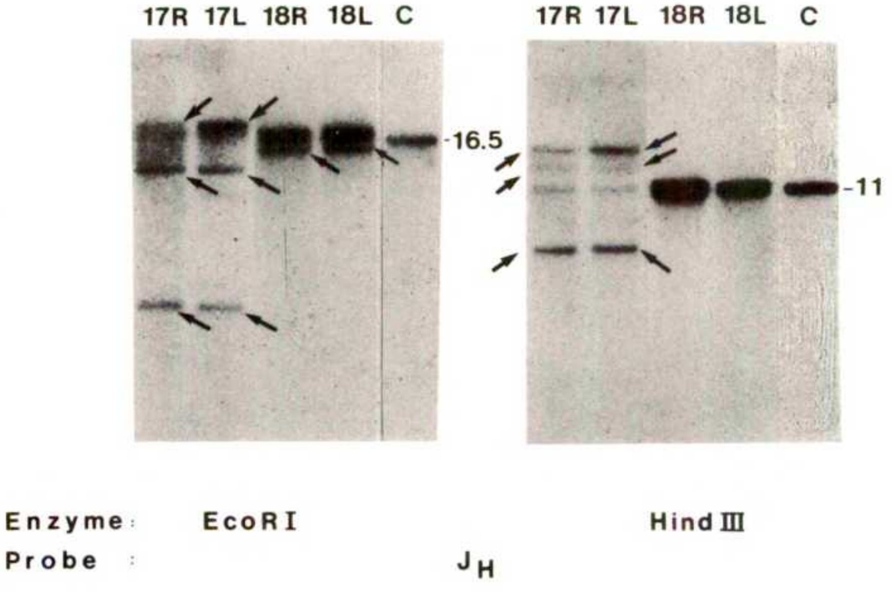

Fig 2. Southern blot hybridization analysis of IgH gene in DNAs extracted from bilateral ocular adnexal monoclonal B cell neoplasms. Lanes C contain human fibroblast DNA. The number above each of the other lanes corresponds to the case number in Table 1, and the letters *R* and *L* indicate right and left, respectively. The DNAs were digested with *Eco*RI and *Hind*III and hybridized to a J_H probe. Rearrangement bands are indicated by arrows. Lanes C show the IgH gene germline configuration without rearrangement bands. The bilateral ocular adnexal monoclonal B cell neoplasms occurring simultaneously in each individual exhibited identical clonal IgH gene rearrangement patterns, which suggested that each neoplasm was derived from the identical B cell clone and that they do not represent separate primary neoplasms.

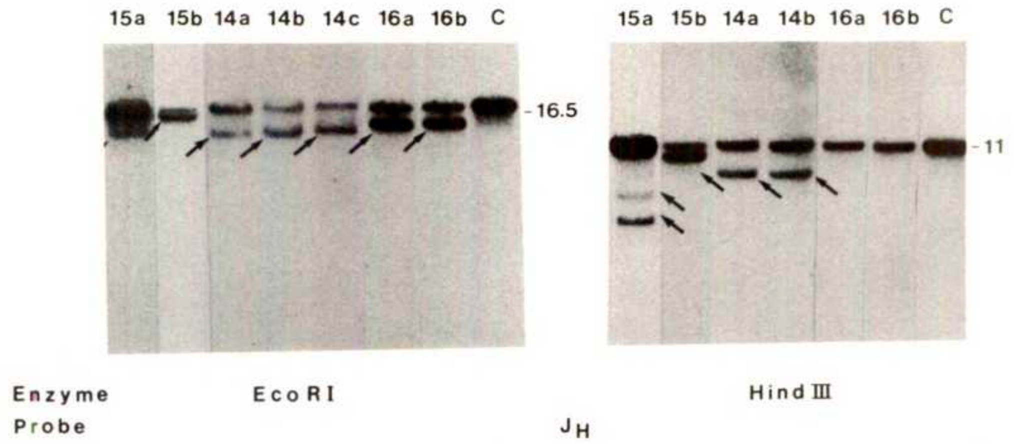

Fig 3. Southern blot hybridization analysis of IgH gene in DNAs extracted from ocular adnexal monoclonal B cell neoplasms and nonocular lymphoid neoplasms occurring concurrently in the same individual. Lanes C contain human fibroblast DNA. The number above each of the other lanes corresponds to the case number in Table 1, and the letters *a*, *b*, and *c* indicate different anatomic sites. The DNA's were digested with *Eco*RI and *Hind*III and hybridized to a J_H probe. Rearrangement bands are indicated by arrows. Lanes C show the IgH gene germline configuration without rearrangement bands. The ocular adnexal and nonocular lymphoid neoplasms occurring concurrently in patient nos. 14 and 16 exhibit identical clonal IgH gene rearrangement patterns which suggested that they are derived from the identical B cell clone. The lymphoid neoplasms occurring in patient no. 15 exhibit distinctive clonal IgH gene rearrangement patterns.

clonal B cell populations by molecular genetic analysis that were not recognizable by morphological examination and immunophenotypic analysis.

Eventual patient outcome. Thirteen of the 18 patients had an ocular adnexal lymphoid neoplasm that we determined to be monoclonal B cell in nature by immunophenotypic analysis. Five of these 13 patients were asymptomatic, without evidence of ocular or nonocular lymphoid neoplasia at 12, 14, 28, 36, and 39 months, respectively. Four patients were alive with evidence of systemic lymphoma at 12, 14, 24, and 26 months, respectively. Three patients died of systemic malignant lymphoma 15, 16, and 29 months after their presentation with an ocular adnexal lymphoid neoplasm. One patient died of metastatic bronchogenic carcinoma 46 months after presentation but had no evidence of ocular or nonocular lymphoid neoplasia at postmortem examination.

Five of the 18 patients had an ocular adnexal lymphoid neoplasm that we determined to be polyclonal by immunophenotypic analysis. The neoplasms in two of these patients were also polyclonal at the molecular genetic level, the

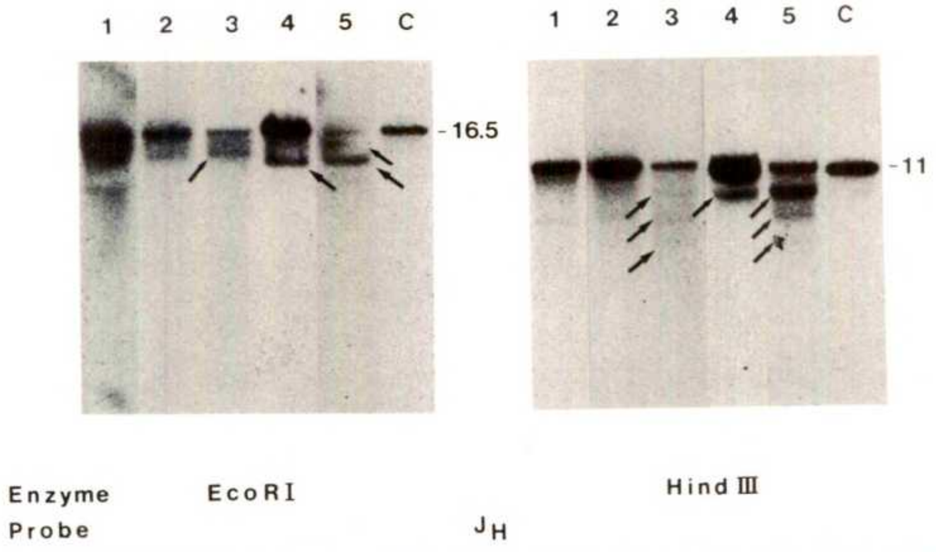

Fig 4. Southern blot hybridization analysis of IgH gene in DNAs extracted from benign, polyclonal ocular adnexal pseudolymphomas. Lanes C contain human fibroblast DNA. The number above each of the other lanes corresponds to the case number in Table 1. The DNAs were digested with *Eco*RI and *Hind*III and hybridized to a J_H probe. Lanes C show the IgH gene germline configuration without rearrangement bands. Case nos. 1 and 2 lack clonal IgH gene rearrangement bands and thus lack detectable monoclonal B cell populations. Case nos. 4 and 5 display clearly visible clonal IgH gene rearrangement bands, which indicate the presence of monoclonal B cell populations. Case no. 3 contains faint, barely perceptible IgH gene rearrangement bands, which suggests the presence of multiple clonal B cell expansions. The clonal B cell populations in case nos. 3 to 5 were not detectable by morphological examination and immunophenotypic analysis.

neoplasm in one patient contained faint oligoclonal bands, and the neoplasms in the two remaining patients contained clearly identifiable clonal B cell expansions by molecular genetic analysis. The two patients with ocular adnexal lymphoid neoplasms that were immunophenotypically polyclonal but monoclonal B cell by molecular genetic analysis were alive, asymptomatic, and without evidence of ocular or extraocular lymphoid neoplasia at 29 and 44 months, respectively. The patient with oligoclonal bands had no evidence of systemic disease after 56 months of follow-up. One of the two patients with a polyclonal lymphoid proliferation was alive and well without evidence of disease at 43 months, whereas the other patient developed a monoclonal B cell proliferation in the contralateral orbit 43 months after the initial presentation.

DISCUSSION

Southern blot hybridization analysis using specific Ig and Tβ gene DNA probes allowed us to determine the lineage and clonality of 20 extranodal lymphoid neoplasms originating and/or presenting in the ocular adnexa of 18 patients and four nonocular lymphoid neoplasms occurring in three of these same patients. The results of molecular genetic analysis were correlated with the results of morphological and immunophenotypic examination of the same samples and with the clinical course of each patient. The results of these studies provide several observations with significant clinical and biologic implications concerning extranodal lymphoid neoplasia. First, ocular adnexal lymphoid neoplasms composed of monomorphic collections of small cytologically mature appearing lymphocytes exhibiting Ig light-chain isotypic exclusion clearly represent clonal B cell expansions. This is true for lymphoid neoplasms originating in and remaining limited to the ocular adnexa as well as those associated with disseminated systemic lymphoma. Second, simultaneous bilateral ocular adnexal monoclonal B cell neoplasms may be derived from the identical B cell clone and do not necessarily represent separate primary tumors. Third, the ocular adnexal and nonocular lymphoid neoplasms occurring concurrently in the same individual may be derived from the same B cell clone. Fourth, many ocular adnexal lymphoid neoplasms currently classified as benign pseudolymphomas may contain clonal B cell expansions demonstrable by molecular genetic analysis, which are not recognizable by histopathologic and/or by immunophenotypic examination. This finding does not necessarily imply that these pseudolymphomas are in reality malignant lymphomas because clonality cannot be equated with malignancy. However, this finding does support the contention that monoclonal B cell lymphomas may arise in extranodal polyclonal pseudolymphomas.

The determination of the benignancy or malignancy of an extranodal lymphoid neoplasm has been based upon traditional, long-established morphological criteria.[32,33] It is generally accepted that cellular polymorphism and cytological maturity favor benignancy whereas monomorphism and cytological atypia favor malignancy.[3] Extranodal small lymphocytic neoplasms have been traditionally classified as benign pseudolymphomas because of their apparent cytological maturity and lack of atypia by morphological examination.[32,33] However, many extranodal small lymphocytic neoplasms resemble nodal small (well-differentiated) lymphocytic lymphomas morphologically.[3] Moreover, the cells frequently express low-density monotypic sIg and the T1 antigen,[5,34] similar to the cells of nodal small (well-differentiated) lymphocytic lymphomas.[35-37] These observations have led some observers to suggest that many extranodal small lymphocytic neoplasms actually represent small (well-differentiated) lymphocytic lymphomas.[2]

Molecular genetic analysis is a considerably more objective and accurate approach than morphological examination and immunophenotypic analysis in determining the lineage and clonality of lymphoid populations. Our molecular genetic studies demonstrate that lymphoid neoplasms resembling nodal small (well-differentiated) lymphocytic lymphomas morphologically and immunophenotypically but that are localized to the ocular adnexa exhibit clonal IgH gene rearrangements. These studies also demonstrate that the various ocular adnexal and nonocular lymphoid neoplasms resembling nodal small (well-differentiated) lymphocytic lymphomas morphologically and immunophenotypically that occur as simultaneous bilateral ocular adnexal or concurrent ocular and nonocular lymphoid neoplasias in a single patient nearly always express an identical pattern of clonal IgH gene rearrangements, which suggests that they are derived from one distinctive B cell clone. This is strong and compelling evidence that the multiple lymphoid tumors exhibiting the small (well differentiated) lymphocytic lymphoma morphology occurring in each individual represent one malignant lymphoma that disseminated. These findings support our contention that extranodal lymphoid neoplasms morphologically identical to nodal small (well-differentiated) lymphocytic lymphoma legitimately fall into the spectrum of small (well-differentiated) lymphocytic lymphomas. Taken together, the clinical, morphological, immunophenotypic, and now the molecular genetic evidence suggests that small lymphocytic neoplasms that occur in extranodal sites such as the ocular adnexa represent the extranodal counterpart of nodal small (well-differentiated) lymphocytic lymphomas whether they are localized to an extranodal site or are disseminated.

It can be correctly argued that the molecular genetic determination of B cell clonality does not in itself imply malignancy, ie, complete transformation, nor does it necessarily imply that a lymphoid neoplasm will behave in a clinically aggressive manner. Nodal small (well-differentiated) lymphocytic lymphomas consistently exhibit clonal IgH gene rearrangements, but they are often clinically indolent and only slowly progressive.[38] The results of the studies presented here suggest that the majority of ocular adnexal small lymphocytic lymphomas remain limited to the ocular adnexa and also are clinically indolent; only a minority are associated with disseminated systemic lymphoma. The ability to predict which small lymphocytic lymphomas will be clinically aggressive may have to await recognition of oncogenic marker(s) associated with their malignant transformation.

It is well known that benign pseudolymphomas occur in each of the anatomic sites in which malignant B cell lymphomas also originate.[1] For example, chronic lymphocytic thyroiditis is commonly seen in association with malignant lymphomas that originate in the thyroid gland.[39] In addition, Sjögren's syndrome and benign lymphoepithelial lesions often antedate the development of monoclonal B cell lymphomas in the salivary glands.[40] Therefore, it has been suggested that extranodal benign pseudolymphomas may serve as precursor lesions and/or be associated with the development of extranodal malignant lymphoma in some instances.[41] Support for this hypothesis comes from the recognition of "clonal proliferation centers" within benign lymphoepithelial lesions of the salivary gland.[42]

In this respect, one of the most interesting observations of this study was that many ocular adnexal lymphoid neoplasms classified as benign pseudolymphomas may contain clonal B cell expansions that are not recognizable morphologically and/or immunophenotypically. In fact, only two of the five ocular adnexal pseudolymphomas that we investigated lacked clonal B cell populations and were truly polyclonal. These results strongly suggest that clonal B cell expansions may commonly occur within benign extranodal pseudolymphomas. This finding provides considerable further support for the contention that monoclonal B cell lymphomas may originate in benign extraodal pseudolymphomas. However, longitudinal multiparametric studies of patients with extranodal lymphoid neoplasms are necessary to substantiate this hypothesis.

It could be argued that we did not identify these small clonal B cell populations immunophenotypically because they were selectively lost during their extraction from the tissue samples. However, our cell isolation techniques have been successfully used in the phenotypic identification of clonal B cell populations for several years and are carefully monitored at each step to avoid cell loss. Mononuclear cell viability was consistently $\geq 90\%$, and appreciable cell loss was not noted in any of the cases included in this study. Moreover, molecular genetic analysis of 45 benign, hyperplastic lymph nodes handled in an analogous manner revealed an occult clonal B cell population in only one instance (data not shown). Therefore, we do not believe that the discrepancy between morphological examination, immunophenotypic analysis, and molecular genetic determination of clonality resides at the technical level. Rather, we believe that the molecular genetic detection of these B cell clones is due to the exquisite sensitivity of the Southern blot technique, which previously has been shown to detect DNA that is representative of $\leq 5\%$ of the total DNA in a given sample.[10,13]

We previously reported that ocular adnexal pseudolymphomas commonly contain increased numbers of T4 antigen–positive T cells.[43] We previously had not been able to determine the clonality of this cell population because there is no reliable phenotypic marker to determine T cell clonality. However, we were now able to investigate the clonality of this T cell population by using a specific DNA probe that detects clonal $T\beta$ gene rearrangements. Using this approach, we demonstrated that the large number of T cells present in some ocular adnexal lymphoid neoplasms do not exhibit clonal $T\beta$ gene rearrangements and therefore do not represent clonal T cell proliferations.

It should be clear from the studies presented here that molecular genetic analysis provides a useful, accurate, and objective approach to evaluate the lineage and clonality of extranodal lymphoid neoplasms. This approach should be useful in identifying patients with extranodal monclonal B cell proliferations that cannot be identified by morphological examination and/or by immunophenotypic analysis and that have been designated indeterminate by some investigators.[44] For example, immunogenotypic analysis should be particularly helpful in examining small biopsy samples of extranodal lymphoid proliferations, specimens in which a large inflammatory response masks a malignant lymphoma, and specimens in which there is only partial involvement by malignant lymphoma. This approach will also be useful in determining clonality in extranodal lymphomas that exhibit atypical histopathology, ie, contain prominent germinal centers, a polymorphous cellular component, or cells that are cytologically mature. The identification of patients with extranodal monoclonal B cell proliferations should provide useful prognostic information and may be helpful in identifying patients in which there is a possibility of conversion from a small (well-differentiated) lymphocytic lymphoma to a diffuse aggressive lymphoma. Furthermore, the identification of a clonal B cell population by its distinctive pattern of Ig gene rearrangements provides a useful genetic marker with which to follow a patient's future lymphoid proliferations.

However, our demonstration of the presence of clonal B cell populations in some morphologically benign and immunophenotypically polyclonal pseudolymphomas also raises important clinical questions concerning the role of antigen receptor gene rearrangement analysis in the diagnosis of malignant lymphoid neoplasia. None of the three patients in this series carrying such lesions developed overt clinical evidence of lymphoid malignancy at 29, 44, and 56 months. We have previously also identified patients with the acquired immunodeficiency syndrome–related complex without clinical evidence of lymphoid malignancy who have hyperplastic lymph nodes containing clonal B cell expansions demonstrable by Southern blot hybridization analysis.[45] These results support a widespread belief that clonality should not be equated with malignancy. It will be necessary to investigate additional cases of extranodal pseudolymphoma and to carefully monitor such patients longitudinally to determine the clinical and biologic significance of these subclincial clonal B cell populations. Nonetheless, the results of these studies demonstrate the value of Southern blot hybridization analysis for clonal Ig and $T\beta$ gene rearrangements in the diagnosis, classification, and investigation of extranodal lymphoid neoplasms originating and/or presenting in the ocular adnexa.

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Soluble Interleukin-2 Receptors in the Sera of Patients With Hairy Cell Leukemia: Relationship With the Effect of Recombinant α-Interferon Therapy on Clinical Parameters and Natural Killer In Vitro Activity

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In this study we provide evidence that the sera of patients with hairy cell leukemia (HCL) contain a factor that can prevent the binding of a monoclonal antibody specific for interleukin-2 receptor (IL-2R) to its target. This factor corresponds to the soluble form of IL-2R (sIL-2R), as assessed by a specific enzyme-linked immunosorbent assay test, and appears to be released by neoplastic hairy cells. The serum sIL-2R levels were very high at diagnosis and significantly reduced during recombinant α-interferon (rIFNα2) therapy. Values of sIL-2R appeared to be inversely related to the natural killer in vitro function displayed by peripheral blood mononuclear cells from the same patients. The presence of sIL-2R in the serum of patients with HCL might be involved in the impairment of cell-mediated immunity observed in these patients and could represent a valuable marker for monitoring different phases of the disease and for modulating IFN therapy.

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HAIRY CELL LEUKEMIA (HCL) is a chronic lymphoproliferative disorder characterized by splenomegaly and pancytopenia. The bone marrow is infiltrated by atypical cells showing a peculiar hairy morphology that can usually be found also in the peripheral blood in variable numbers.[1,2] Hairy cells (HC) belong to the B cell lineage and characteristically express the interleukin-2 receptor (IL-2R) on their membranes.[3] This molecule plays a crucial role in lymphocyte proliferation and is normally expressed on the surface of activated T and B cells.[4] It has been recently demonstrated in both human and experimental animals that in vitro activated normal lymphoid cells can release a soluble form of IL-2R (sIL-2R).[5-7]

Contrasting data have been provided on the role of IL-2 in the proliferation of HC in vitro.[3,8] There are some data suggesting the possibility that impairment of the IL-2/IL-2R mechanism might be related to immunologic abnormalities observed in other lymphoproliferative disorders.[9,10]

A severe impairment of natural killer (NK) activity has been extensively demonstrated in HCL.[11-16] The defect of cytotoxic function could contribute to the increase susceptibility of HCL patients to infections, which represent the primary cause of mortality in this disease.[17,18] One interesting possibility in HCL is that the IL-2R might adsorb and block IL-2, thereby making it unavailable for stimulating NK cells.[19] Such a mechanism would be greatly facilitated by the release of sIL-2R from HC and its diffusion through the plasma and internal milieu. To test this hypothesis, we looked for the presence of sIL-2R in the sera of patients with HCL by using two different techniques: a competitive binding immunocytochemical assay and a recently standardized enzyme-linked immunosorbent assay (ELISA) test. We tested eight patients with HCL at diagnosis and during their clinical and hematologic improvement after therapy with α-interferon (rIFNα2), which is highly effective in HCL.[20] The findings have been compared with the analysis of NK cell in vitro function.

MATERIALS AND METHODS

Patients and sera. Eight patients with HCL, one female and seven males (average age, 62 years, Table 1), were studied. The main clinical and laboratory data of patients at diagnosis and their variations during rIFNα2 therapy are shown in Fig 1. The diagnosis of HCL was based on clinical, morphological, cytochemical, and immunophenotypic criteria.[1-3] In all patients bone marrow involvement was confirmed by the immunohistochemical demonstration of HC.[21] All patients had severe neutropenia ($<0.5 \times 10^9$/L) and entered a therapeutic protocol with rIFNα2 (Schering Corp, Kenilworth, NJ). None of the patients had splenectomy nor underwent any other treatment before rIFNα2 therapy. Treatment started in November 1984 for the first three patients, whereas the other patients started the therapeutic regimen later. The protocol consisted of 2×10^6 IU/m^2 of rIFNα2 injected subcutaneously three times a week for 12 months. Informed consent was obtained from all patients, who had been advised of procedures and attendant risks. Samples of serum were collected from all patients at diagnosis and at different times during rIFNα2 therapy and evaluated for the presence of sIL-2R in conjunction with clinical and phenotypical assessment of the disease status. Sera from 40 normal age-matched subjects were used as control.

Competitive binding immunocytochemical assay to detect sIL-2R. The exquisitely sensitive alkaline phosphatase anti–alkaline phosphatase (APAAP) technique[22] was used to detect the specific immunostaining of anti–IL-2R+ target cells and reveal the inhibitory capability of sera. Hightly enriched T cells obtained by E rosetting sedimentation from the peripheral blood mononuclear cells (PBMC) of normal donors were activated in vitro for 72 hours with 2 μg/mL Phytohaemagglutinin (PHA-M) (Difco Detroit) and used as standard IL-2R+ targets. The anti–IL-2R monoclonal antibody (MoAb) was purchased from Becton Dickinson, Sunnyvale, CA (lot no. H0716).

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Table 1. Semiquantitative Evaluation of the Inhibitory Capability of Patients' Sera on the Binding of Anti–IL-2R Antibody to Activated T Cells

Patient No.	Age	Sex	Dilutions of Patient's Serum 1:1	1:2	1:5	1:10*
1	44	M	+	+	+	−
2	64	F	+	+	+	−
3	64	M	+	+	−	−
4	70	M	+	+	+	−
5	75	M	+	+	+	−
6	57	M	+	+	+	−
7	71	M	+	+	−	−
8	57	M	+	+	+	+
Normal (ten cases)			−	−	−	−

Patients' sera were obtained at diagnosis. + complete inhibition of anti-IL2R binding to activated T cells.

*One microliter anti–IL-2R antibody (1:100 dilution) was mixed with different volumes of patient's serum (1 μL, 2 μL, 5 μL, 10 μL), the final volume adjusted to 10 μL with PBS.

Anti–IL-2R antibody was first diluted 1:100 in phosphate-buffered saline (PBS). One microliter of this solution was then added to 10 μL PBS (obtaining the 1:1,000 final working dilution used to detect positive staining) or to 10 μL of sera at different dilutions (1:1, 1:2, 1:5, 1:10, 1:20). The mixtures containing antibody and sera were allowed to incubate for 20 minutes at room temperature before the immunocytochemical staining.

Acetone-fixed cytocentrifuged preparations of activated T cells were incubated for 30 minutes with anti–IL-2R antibody diluted 1:1,000 in PBS or serum at various dilutions. After washing, the preparations were reacted for 30 minutes with a goat antimouse Ig "bridge" antibody before the incubation with the APAAP complex (purchased from Dako, Copenhagen). Finally, alkaline phosphatase was revealed cytochemically by means of naphthol AS-BI phosphate (Sigma Chemical Co, St Louis) as the substrate and hexazotized neufuchsin (Serva, Heidelberg, FRG) as the chromogen.

The immunostained preparations were evaluated as positive or negative by direct microscope observation and semiquantitative scoring of the number of IL-2R+ cells in the preparations. Preparations were considered negative when less than 1% of the cells showed a faint positivity.

Additional IL-2R+ targets were cryostat sections of HCL bone marrow trephine biopsy material and samples of Hodgkin's disease–involved lymph nodes. Both presented large numbers of IL-2R+ cells, HC, and Reed-Sternberg cells,[23] respectively.

Controls. Three different types of controls were performed. First, the possible inhibition of binding of MoAbs other than anti–IL-2R (Leu-4, Leu-1, and anti–HLA-DR, from Becton Dickinson) by normal and patient's sera was investigated in parallel tests. The antibodies used as controls were either of the same class as anti–IL-2R (IgGl, Leu-4) or of different classes (IgG2a, anti–HLA-DR and Leu-1)

Second, the possibility that the preincubation of target cells with the sera could prevent the binding of anti–IL-2R antibody was checked to rule out the presence in HCL sera of autoantibodies specific for IL-2R, as found in systemic lupus erythematosus.[24] These antibodies could produce similar inhibition of anti–IL-2R binding to target cells in the same system. Finally, the sera were dialyzed overnight in buffered saline by using standard Visking tubes before testing for immunocytochemical inhibition.

ELISA test for the determination of sIL-2R. Two MoAbs recognizing different epitopes of the IL-2R molecule were used in an ELISA test (Cellfree, T Cell Science, Cambridge, MA). Briefly, sIL-2R available in the samples or standards bind to the polystyrene microtiter wells previously incubated with 100 μL anti–IL-2R MoAb (1 μg/mL). A horseradish peroxidase–conjugated anti–IL-2R MoAb directed against a second epitope on the IL-2R molecule binds to the IL-2R captured by the first antibody and completes the sandwich. After washing to remove the unbound enzyme-conjugated anti–IL-2R MoAb, a substrate solution is added to the wells. The reaction is then stopped and the absorbance determined at 490 nm. A standard curve was prepared by using different sIL-2R concentrations defined in arbitrary units per milliliter (considering 1,000 U/mL the amount of sIL-2R present in 0.1 mL of a T Cell Science reference preparation of supernatant from phytohemagglutinin-stimulated lymphocytes).

Demonstration of sIL-2R release by HC in vitro. HC-enriched populations were obtained in five patients in which, at the time of diagnosis, the frequency of HC was highest. HC-enriched cell suspensions were obtained after removal of T cells according to the method previously described in detail[25] and never contained >1% T cells or monocytes as detected by indirect immunofluorescence with CD3 MoAb and α-naphthyl acetate esterase. The frequency of anti-Tac and tartrate-resistant acid phosphatase–positive cells was >77% and >86%, respectively.

The HC-enriched populations (1×10^4/well) were cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Microbiological Products, Wakersville, MA), penicillin, streptomycin, and L-glutamine in 96-well U-bottomed microtiter plates in a final volume of 200 μL. They were cultured for three or seven days in a 5% CO_2 atmosphere in the presence of medium alone or 12-0-tetradecanoylphorbol-13-acetate (TPA 10 ng/mL/1×10^6 cells; Sigma) and/or B cell growth factor (BCGF, 20% vol/vol 1×10^6 cells; Chemicon, El Segundo, CA). All cultures were performed in triplicate. After the incubation period, the plates were centrifuged, and culture supernatants were harvested to determine the presence of sIL-2R as described earlier. Cellular viability was always >95%.

Evaluation of NK activity. PBMC were obtained from freshly drawn heparinized peripheral blood by centrifugation on a Ficoll/Hypaque gradient. The cells were washed three times and resuspended in RPMI 1640. Adherent cells were removed by two sequential incubations in plastic Petri dishes for 45 minutes at 37°C in an atmosphere of 95% air and 5% CO_2.

NK activity was assessed by lysis of ^{51}Cr-labeled K-562 target cells as previously described.[15] Briefly, target cells were labeled overnight at 37°C in 5% CO_2 with 100 μCi Na $^{51}CrO_4$ (CEA, Saluggia, Italy; IRE Sorin, Biomedica), extensively washed three times before use, and then adjusted to a final concentration of 10^5 cells/mL in RPMI 1640 supplemented with 10% FCS. To perform the test, 100 μL labeled targets (10^4 cells) was cocultured with effector cells for four hours at 37°C in 5% CO_2 at different dilutions to final ratios between effectors and target cells (E/T) of 5:1, 10:1, 20:1, 40:1, and 80:1 in triplicate round-bottomed wells (Limbro Scientific Co, Hamden, CT). Triplicate wells with target cells in medium alone and in detergent were assessed to determine spontaneous and maximum release, respectively. After this incubation, supernatants were harvested and counted in a gamma counter. The mean value of triplicate assays was used to calculate the percentage of cytotoxicity according to the following formula: percent cytotoxicity = (cpm release in test − cpm spontaneous release) × 100/(cpm maximum release − cpm spontaneous release). Spontaneous release from the target cells was always <8%. Nonadherent PBMC from 20 age-matched healthy volunteers were used as control. Data are presented at the standard 80:1 E/T ratio as previously described in detail.[15]

NK cell activity after in vitro incubation with IL-2. The effect of IL-2 on the cytotoxic activity was determined after in vitro boosting. Effector cells (1×10^6/mL) were cultured in RPMI 1640

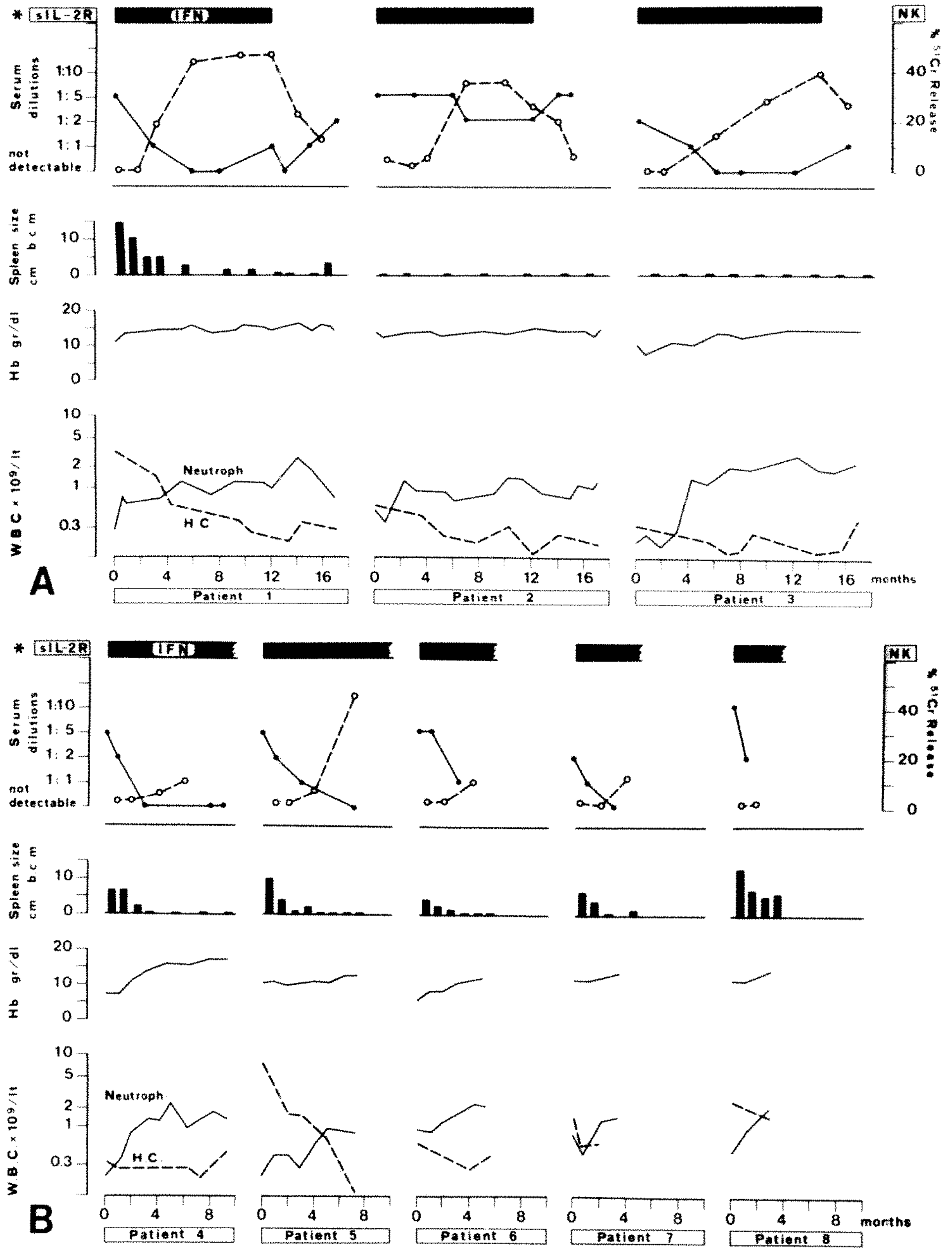

Fig 1. (A) Longitudinal evaluation of clinicohematologic data in relationship with sIL-2R (—●—) levels and NK activity (—O—) of patients with HCL treated with rIFNα2 (case nos. 1, 2, and 3). *Inhibitory capability of HCL sera on the binding of anti–IL-2R antibody to activated T cells (sIL-2R) expressed as maximal serum dilutions at which sIL-2R is detectable. (B) Longitudinal evaluation of clinicohematologic data in relationship with sIL-2R (—●—) levels and NK activity (—O—) of patients with HCL treated with rIFNα2 (cases 4 to 8). *Inhibitory capability of HCL sera on the binding of anti–IL-2R antibody to activated T cells (sIL-2R) expressed as maximal serum dilutions at which sIL-2R is detectable.

medium supplemented with 10% FCS, 4 mmol/L glutamine, penicillin (100 U/mL), and streptomycin (50 mg/mL) and that contained 20% vol/vol lectin-free IL-2 (Lymphocult-LF, Biotest, Frankfurt, FRG) for 72 hours at 37°C in a 5% CO_2 atmosphere. After incubation with IL-2, the cells were washed, resuspended in fresh medium, and immediately tested for cytotoxicity as described earlier. These doses and times were previously found to give the highest increase of NK activity on normal peripheral blood lymphocytes (data not shown). In all experiments effector cells cultured in the same conditions, but without IL-2, were used as controls. To rule out the possibility of a stimulatory effect of the FCS present in the culture medium, appropriate controls with medium alone were performed. In these experiments no appreciable cytotoxic activity was detected. Results are expressed as percentages of ^{51}Cr release (at the 80:1 E/T ratio) by cells cultured with IL-2 with respect to the cells cultured in the same experimental conditions but without IL-2.

RESULTS

When cytospin preparations of activated T cells were stained with anti–IL-2R antibody at a 1:1,000 dilution, the APAAP technique revealed membrane staining in 25% to 50% of the cells. The reactivity was not inhibited by normal sera (ten cases) added in the highest concentrations used in our system (1 μL prediluted anti–IL-2R antibody plus 10 μL serum; final concentration, 1:1,000). On the other hand, all the sera of HCL patients at diagnosis completely inhibited the anti–IL-2R staining (Table 1). The inhibition was seen at 1:1, 1:2, and 1:5 dilutions, and a positive staining appeared again only when anti–IL-2R antibody was mixed with HCL sera at the highest dilutions. Most cases (5/8) showed inhibitory capability at a 1:5 serum dilution, one case at 1:10, and two cases at 1:2 (Table 1).

Table 2. Quantitative Evaluation of sIL-2R in the Sera of HCL Patients at the Time of Diagnosis and During rIFNα2 Therapy

	sIL-2R* (U/mL)				
Patient No.	Before Therapy	3 mo†	6 mo†	12 mo†	15 mo†
1	37,980	5,400	1,880	1,452	10,220
2	26,680	14,973	15,200	6,230	18,570
3	13,370	1,935	1,112	958	2,194
4	20,500	1,885	981	690	
5	18,100	ND	2,748	1,656	
6	48,090	8,095	3,660		
7	16,080	2,232	1,294		
8	26,070	4,875	2,748		

Abbreviation: ND, not done.

*Age-matched normal controls: mean ± SD = 232 ± 118 U/mL; range, 75 to 496 U/mL.

†Months after the beginning of rIFNα2 therapy.

Controls. The sera of patients with HCL did not inhibit the immunostaining of MoAbs other than anti–IL-2R (anti–HLA-DR, Leu-4, and Leu-1) in an identical system using the same (1:1,000) antibody dilution. The binding of anti–IL-2R antibody was not prevented by preincubation of activated T cells with the sera of patients affected with HCL. Overnight dialysis of sera did not modify their inhibitory capability.

ELISA test for the determination of sIL-2R. The presence of sIL-2R in the sera of HCL patients at diagnosis was confirmed by using the enzyme immunoassay (Table 2). In all cases the values were very high (mean ± SD = 25,858 ± 11,846 U/mL; range, 13,370 to 48,090 U/mL) when compared with those observed in age-matched normal controls (mean ± SD = 232 ± 118 U/mL; range, 75 to 496 U/mL).

Detectable levels of sIL-2R were found to be released in the supernatants of cultured HC after stimulation up to seven days. sIL-2R were undetectable in all cases at day 0, ranged between 73 and 1,300 U/mL at day 3, and between 132 and 2,600 U/mL at day 7. Spontaneous sIL-2R release was documented without stimulation in two cases.

sIL-2R serum levels after rIFNα2 therapy. A consistent reduction of sIL-2R levels was observed after rIFNα2 therapy. This was demonstrated by the decrease and eventually the disappearance of the inhibitory effect of sera in the cytochemical competitive assay (Fig 1) as well as by the progressive reduction of values obtained with the ELISA test (Table 2). sIL-2R levels started to rise again after discontinuation of the rIFNα2 therapy as shown in the patients with the longest follow-up (Fig 1 and Table 2). In only one patient who received rIFNα2 for 1 year (case no. 2) were the sIL-2R levels only partially reduced during the course of therapy. This same patient experienced a rather modest clinical response (only a moderate increase of granulocytes and a small reduction of HC levels in the bone marrow). In the other patients the progressive loss of sIL-2R in the serum correlated with clinical improvement, as judged by the decrease of neoplastic cells in peripheral blood and marrow, the reduction of the spleen size in patients with enlarged spleens, and the increase in granulocytes and hemoglobin levels (Fig 1).

Relationship between sIL-2R levels and NK in vitro activity. As shown in Fig 1, the NK in vitro function was severely impaired before therapy in our patients. The cytotoxic ability of patients' mononuclear cells started to increase in the third month of rIFNα2 therapy and reached normal values after 6 months. In the three patients who had already discontinued therapy, rIFNα2 interruption was followed by a new decrease in NK function (Fig 1). Interestingly, the NK function in the HCL patients appeared inversely related to the levels of sIL-2R (Fig 1). Notably, when the sIL-2R levels were high, the NK function was low; when the sIL-2R levels reached their nadir, the cytotoxic ability began to increase.

In vitro effect of IL-2 on NK activity. The stimulatory effect of exogenous IL-2 on the NK in vitro function of PBMC from HCL patients is shown in Table 3. At the time of diagnosis before any treatment, exogenous IL-2 was able to provide a dramatic increase in the cytotoxic in vitro function in all tested patients. It is worth mentioning that at this time the highest levels of sIL-2R were also observed in the patients' serum.

After a 6-month period of therapy with rIFNα2 when sIL-2R levels were significantly decreased, the NK in vitro function was only slightly susceptible to further increase upon IL-2 stimulation in all cases but one (case no. 2). This pattern was similar to that observed in normal subjects.

The immunophenotypic evaluation of NK-related cells when using Leu-7 and Leu-11 MoAbs showed comparable figures at diagnosis and after 6 months of rIFNα2 therapy (Leu-7, 0.311 × 10^9/L ± 0.169 *v* 0.237 × 10^9/L ± 0.207; Leu-11, 0.127 × 10^9/L ± 0.089 *v* 0.107 × 10^9/L ± 0.071)

Table 3. In Vitro Effect of IL-2 on the NK Activity of PBMC From HCL Patients Before and During rIFNα2 Treatment

	Before Treatment			After 6 mo of Therapy		
	^{51}Cr Release (%)			^{51}Cr Release (%)		
Patient No.	Baseline Value	After IL-2 Activation	Variance	Baseline Value	After IL-2 Activation	Variance
2	5.6	36.0	30.4	21.8	48.8	27.0
3	6.8	39.5	32.7	17.3	21.6	4.3
4	3.7	25.2	21.5	12.0	12.0	0
5	8.0	57.3	49.3	42.5	50.7	8.2
Controls (n = 5)						
	44.6 ± 8.6*	57.7 ± 6.4*	13.1			

For NK activity, a standard assay was performed at an 80:1 E/T ratio.

*SD.

(mean ± SD). This finding suggests that the variations in NK activity are not primarily produced by fluctuations in NK cell numbers.

DISCUSSION

In this paper we provide evidence that the serum of patients with HCL contains high levels of sIL-2R. This was suggested by the selective inhibition of anti–IL-2R antibody binding to activated T cells that was exerted by patients' sera and confirmed by a specific ELISA test. The inhibitory factor corresponding to the sIL-2R should be released in vivo by HC, as suggested by experiments performed in vitro. T cells have been demonstrated to secrete sIL-2R after activation in vitro[5] and might then represent an alternative or supplementary source of sIL-2R in vivo in our patients. Nevertheless, no evidence has been reported of increased T cell activation in HCL. In addition, the sIL-2R levels rarely exceed 2,000 to 4,000 U/mL in other diseases where T cell activation is commonly present such as viral hepatitis, infectious mononucleosis, sarcoidosis, and Hodgkin's disease.[26,27]

Using both methods we could demonstrate that the sIL-2R levels in our patients' sera dramatically decreased after a few months of rIFNα2 therapy. In most patients the decrease in sIL-2R levels corresponded to the clinical recovery and the improvement of erythromyelopoiesis. However, a small number of residual HC could be detected in the bone marrow of all patients, even at the maximum therapeutic effect of rIFNα2 (data not shown), which could account for the observed lack of complete normalization of sIL-2R levels in peripheral blood (Table 2). In addition, the levels of sIL-2R in the serum of HCL patients and the NK in vitro function of PBMC appeared inversely related.

These findings have a series of relevant implications. First, the observed impairment of cell-mediated immunity in HCL[17,18] might be correlated with the overproduction and accumulation of sIL-2R in the serum. As a consequence, the excess sIL-2R released in the serum in vivo might bind IL-2 and block the IL-2/IL-2R modulation necessary for effective cellular proliferation.[28,29] This interpretation is supported by the recent report that sIL-2R released by activated T cells maintain the capability of binding IL-2.[30]

The putative defect of IL-2 at serum level may be particularly important for the NK system in HCL patients where a severe impairment of the NK function has been extensively demonstrated.[11-16] Supportive evidence of the relevance of IL-2 in the mechanisms of the impaired cytotoxic response in HCL is provided by the fact that the addition of exogenous IL-2 to short-term cultured PBMC from HCL patients can restore their NK activity (Table 3).[14,15]

The significant increase of NK function observed during IFN therapy and corresponding to the decrease of sIL-2R levels (Fig 1 and Table 2) raise the question of the relationship between IFN therapy and its biologic and clinical effects. The mechanisms involved in the therapeutic actions of IFN on HCL and other neoplasms are still controversial. It is not established whether IFN directly exerts a cytotoxic effect on neoplastic cells, for example, by inhibition of BCGF-induced proliferation of HC.[31] Alternatively, IFN could indirectly reduce the neoplastic mass by increasing the susceptibility of HC via the modulation of membrane antigens, including HLA-DR determinants,[32] or by the stimulation of immunologic defenses (especially the NK system).[33] The observed lack of timing between the restoration of NK activity and HC decrease in treated patients does not support this latter possibility. This is also suggested by the recent data on the absence of cytotoxic activity of NK cells from HCL patients on their own neoplastic cell population.[34] All these findings favor the hypothesis that the increase in cytotoxic activity observed in HCL after α-IFN therapy represents the effect of a nonspecific enhancement of the host immune system.

The only exception in our study was represented by patient no. 2 in whom high serum levels of sIL-2R were detectable during the entire follow-up of the treatment. A limited clinical response was obtained in this patient, but NK activity significantly recovered during rIFNα2 therapy (Fig 1). This discrepancy may be tentatively explained by differences in IL-2–binding capacity of soluble receptors and/or by a direct activation of NK cells by interferons independent of IL-2 availability. This latter possibility, however, is unlikely because exogenous IL-2 also produced a significant stimulation of in vitro NK activity after 6 months of therapy.

All these considerations taken together suggest that the evaluation of sIL-2R in the serum, beside its relevance to the comprehension of immunologic phenomena taking place in HCL, may be important for monitoring the disease and particularly the response of different patients to interferon therapy. Longitudinal studies are now in progress to verify this possibility.

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Synergistic Action of Calcium Ionophore A23187 and Phorbol Ester TPA on B-Chronic Lymphocytic Leukemia Cells

By Hans G. Drexler, Malcolm K. Brenner, Elaine Coustan-Smith, R. Gitendra Wickremasinghe, and A. Victor Hoffbrand

The peripheral blood mononuclear cells from 16 patients with B-chronic lymphocytic leukemia (B-CLL, n = 13), B-prolymphocytic leukemia (B-PLL, n = 2) or hairy cell leukemia (n = 1) were incubated in the presence of the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) and the calcium ionophore A23187. A synergy between these inducers was found with respect to morphological changes and B cell proliferation and differentiation. A23187 used alone did not activate the cells. B-CLL cells treated with the double stimulus acquired a plasmacytoid morphology, showed significantly higher incorporation of ^{3}H-thymidine and ^{3}H-uridine, and produced significantly higher amounts of monoclonal immunoglobulin compared with the same cells exposed to either of the inducers alone. These results indicate that phorbol ester and calcium ionophore act synergistically on B-CLL cells to induce proliferation and differentiation. B-PLL cells responded more vigorously to the signals provided by TPA and A23187. Previous studies showed that TPA and A23187 can mimic the two physiological second messengers diacylglycerol and inositol trisphosphate in the transduction of signals leading to cell activation, proliferation, and differentiation in normal B cells. The present findings suggest that the capacity of B-CLL and B-PLL cells to differentiate in response to signals of the second messenger pathway is intact.

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CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) is, in most cases, a monoclonal expansion of neoplastic B cells in which differentiation blockage appears to be a basic defect leading to a clonal excess of these cells. Release of this blockage might have therapeutic benefits. Numerous studies have shown that the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) can stimulate differentiation of B cell CLL (B-CLL) cells in vitro into more mature forms[1] and elicit a variety of biologic responses. TPA-treated cells showed altered cell morphology associated with a more differentiated state,[2] and TPA alone induced the secretion of idiotypically identical immunoglobulin (Ig) and caused characteristic changes in the expression of B cell–associated surface antigens.[3]

Recent studies have provided new insights into the molecular basis of signal transduction triggering cellular activation and proliferation. The key event in this signal transduction pathway is the hydrolysis of membrane-associated inositol phospholipids that leads to the generation of two products that act as second messengers of cell activation. Cell activation is initiated in T cells by interaction between an antigen associated with a major histocompatibility complex (MHC) molecule and the T cell receptor[4] and in B cells by cross-linking of receptor immunoglobulin by antigen or antireceptor antibodies.[5] This receptor-ligand interaction causes the hydrolysis of phosphatidyl inositol bisphosphate located in the cell membrane to diacylglycerol and inositol trisphosphate.[6] These two second messengers trigger two parallel pathways that act in concert. Inositol trisphosphate mediates the mobilization of intracellular free calcium by release of Ca^{2+} from intracellular stores, whereas diacylglycerol directly activates protein kinase C, a key calcium-dependent enzyme in cell activation. The subsequent protein kinase C–mediated protein phosphorylation leads to alterations in ion flux that are manifested in membrane depolarization and an increase of intracellular pH.[4,5] These events seem to be causally related to cellular activation and growth.[4]

The intracellular signals triggered by inositol trisphosphate and diacylglycerol can be independently mimicked by calcium ionophores (such as A23187) and phorbol esters (such as TPA), respectively. A23187 causes Ca^{2+} mobilization (calcium influx or transport from intracytoplasmic calcium stores). EDTA abrogates the effect of A23187.[7] TPA, having a diacylglycerol-like structure, substitutes for diacylglycerol rather than by inducing the formation of diacylglycerol. TPA increases the affinity of protein kinase C

Table 1. Clinical Data on Patients Studied

Patient No.	Diagnosis	Age	Sex	WBC (× 10^9/L) at Time of Study	Treatment
1	B-CLL	56	M	88	COP
2	B-CLL	72	F	187	None
3	B-CLL	69	M	213	None
4	B-CLL	70	F	25	None
5	B-CLL	65	M	164	P, Cbl
6	B-CLL	63	M	23	None
7	B-CLL	84	F	40	None
8	B-CLL	64	F	98	None
9	B-CLL	65	F	28	None
10	B-CLL	65	F	46	None
11	B-CLL	61	M	17	None
12	B-CLL	56	F	32	None
13	B-CLL	72	M	28	None
14	B-PLL	77	M	32	None
15	B-PLL	82	M	67	None
16	HCL	62	F	28	None

Abbreviations: C, cyclophosphamide; O, Oncovin; P, prednisolone; Cbl, chlorambucil.

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Table 2. Surface Marker Phenotypes of Patients Studied (Before T Cell Depletion)

			Patient Nos.															
Reagent	Reactivity	CD*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RFDR2	HLA-DR (class II antigen)	—	91†	83	95	89	97	74	84	87	80	90	69	64	90	83	88	82
RFAL3	Common ALL antigen	CD10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RFB4	Pan-B	CD22	85	0	0	0	94	0	78	0	2	70	74	49	0	44	81	77
RFB6	C3d receptor	CD21	90	6	92	5	95	70	79	1	70	75	74	49	89	43	80	1
RBF7	Pan-B	CD20	90	84	94	83	96	72	80	80	78	76	78	52	90	88	81	78
SmIgM/G	Ig heavy chain	—	86	82	nd‡	80§	96	70	nd‡	80	nd‡	70	77	50	90	73	98	63§
SmIgκ/λ	Ig light chain	—	κ	κ	nd‡	κ	κ	nd‡	nd‡	κ	nd‡	κ	κ	nd‡	κ	κ	κ	λ
RFT1	Pan-T	CD5	96	92	94	91	98	85	95	93	95	85	71	77	94	11	98	9
RFT11	E rosette receptor	CD2	8	2	0	7	2	17	22	10	18	14	19	25	5	10	10	10
RFT12	Pan-T	CD6	95	96	93	6	97	84	95	86	94	15	85	nt	93	10	95	10
FMC7‖	Mature B cells	—	4	3	0	0	0	2	1	2	0	6	0	61	82	83	84	56
Tac	IL-2 receptor	CD25	nt	0	81	2	0	nt	0	0	53	91	0	nt	nt	2	91	94

Abbreviations: nt, not tested; nd, not detectable; ALL, acute lymphocytic leukemia; SmIg, surface membrane immunoglobulin; IL-2, interleukin 2.

*Cluster of differentiation group according to First and Second International Workshop.

†Percentage of positive cells.

‡Surface Ig expression not detectable; monoclonality could be shown in the immunoglobulin ELISA assays (patient no. 6, IgMκ; patient no. 7, IgMλ; patient no. 9, IgMλ; patient no. 12, IgMκ).

§Patient nos. 4 and 16 were monoclonal for IgG heavy chain (all other cases monoclonal for IgM).

‖"Marker" of B-PLL and HCL cells.

for calcium[8] but does not provoke a significant increase in Ca^{2+}.[9]

TPA and A23187 act synergistically in activating normal mouse and human B cells.[9-11] However, comparatively little is known of their effects on B-CLL cells. To explore the possibility of inducing differentiation in CLL cells, we studied the synergism between TPA and A23187 with respect to various aspects of B cell activation, including RNA synthesis measured by the uptake of ^{3}H-uridine and DNA synthesis measured by ^{3}H-thymidine uptake, which accompany morphological changes and differentiation to Ig-producing cells.

MATERIALS AND METHODS

Patients. Peripheral blood samples were obtained from 13 patients with B-CLL, two patients with prolymphocytic leukemia (B-PLL), and one patient with hairy cell leukemia (HCL). Diagnosis was based on typical clinical, morphological, cytochemical, and immunologic features. Clinical characteristics of the cases studied are outlined in Table 1. Mononuclear cells from the peripheral blood of four healthy donors were also investigated.

Phenotyping of cells. Mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo) density gradient centrifugation and analyzed by routine leukemia phenotyping.

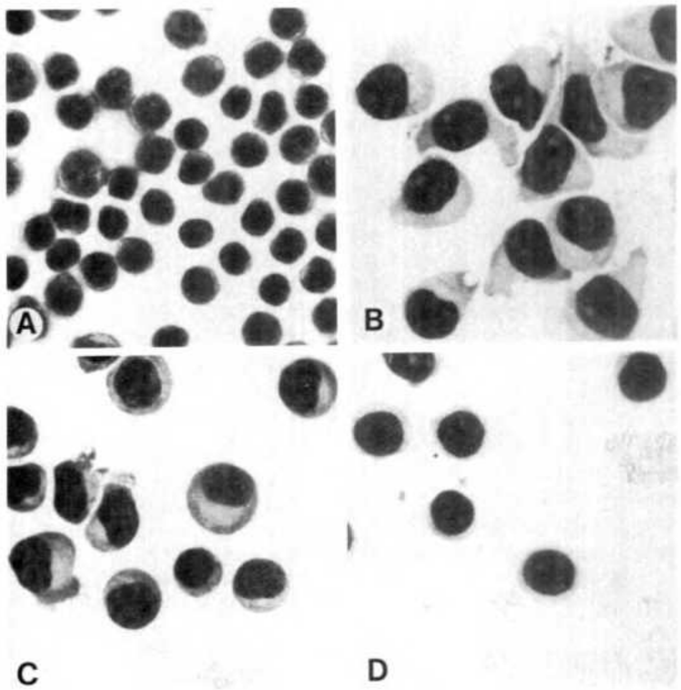

Fig 1. Morphology of B-CLL cells treated with TPA, A23187, or a combination of TPA plus A23187 (May-Grünwald-Giemsa stained; original magnification × 1,000; current magnification × 440). (A) Cells at day 0 (typical morphology of small, round B-CLL cells with scanty cytoplasm). (B) Cells after 72 hours of treatment with 2 × 10^{-8} mol/L TPA (enlarged, irregularly shaped cells with clear cytoplasm). (C) Cells after 72 hours of treatment with 2 × 10^{-8} mol/L TPA plus 0.5 μmol/L A23187 (enlarged, round to oval cells with basophilic cytoplasm, clear perinuclear zone, and eccentrically located nucleus). (D) Cells after 72 hours of treatment with 0.5 μmol/L A23187 (cells are enlarged but remained round with scanty, clear cytoplasm).

Direct and indirect immunofluorescence was performed by using the microtiter plate system as described in detail earlier.[12] Surface Ig expression was determined by direct immunofluorescence tests with fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat antihuman Ig-specific antisera (Southern Biotechnology, Birmingham, AL). κ or λ chain expression was determined by double labeling with specific FITC- and TRITC-conjugated reagents. After incubation with specific monoclonal antibodies, cells were stained with FITC- or TRITC-conjugated goat antimouse Ig reagents. Monoclonal antibodies against the FMC7 and Tac antigens and reagents of the RF-series were kindly provided by Dr Jun Minowada (Fujisaki Cell Center, Okayama, Japan) and Professor George Janossy (Royal Free Hospital, London), respectively. The composite surface marker phenotypes of the patients studied are listed in Table 2.

Separation of cells. After Ficoll separation of mononuclear cells, plastic-adherent cells were depleted by incubation of the cell suspension on Nunc tissue culture plates (Roskilde, Denmark) for 90 minutes at 37°C. The resulting cell population was T cell depleted by rosetting for 45 minutes at 4°C with neuraminidase-treated sheep RBC (TCS, Berkshire, UK). Nonrosetting cells (B lymphocytes) were harvested from the interface of a second Ficoll gradient. The numbers of residual monocytes and T cells were determined by labeling of this population with MCS-2 (CD13), MY4 (CD14), or Leu-M3 (CD14) and Leu-4 (CD3) as described earlier.

Cell culture and in vitro stimulation. Cells were cultivated in RPMI 1640 medium (FLow Laboratories, Rickmansworth, UK) supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Sussex, UK), penicillin/streptomycin (GIBCO, Uxbridge, UK), and L-glutamine (GIBCO) at a cell concentration of 2×10^6/mL in flat-bottomed 96-well microwell plates (Nunc) in a humidified 5% CO_2 atmosphere at 37°C. The cells were continuously incubated (without further addition or exchange of medium) for six days and harvested at 24-hour intervals. The following reagents were added: (*a*) none (control); (*b*) TPA (Sigma Chemical Co, Dorset, UK) at 2×10^{-8} mol/L; (*c*) TPA (2×10^{-8} mol/L) plus A23187 (Sigma) in various concentrations; and (*d*) A23187 at 0.5 µmol/L. In preliminary experiments it was found that concentrations of A23187 higher than 1.0 µmol/L were cytotoxic; the optimal range was 0.3 to 0.7 µmol/L A23187.

Morphological evaluation. Morphological changes were examined on cytocentrifuge slides (Shandon-Elliott, Sewickley, PA) prepared from all cell suspensions and stained with standard May-Grünwald-Giemsa solution. Adherence of cells to culture wells and formation of clusters were visually assessed in the microtiter plates under an inverted phase-contrast microscope.

Analysis of Ig production. The amount of IgM or IgG produced was measured by an enzyme-linked immunosorbent assay (ELISA). Microtiter plates (MicroElisa, Dynatech, Billinghurst, UK) were precoated overnight with rabbit antihuman IgM (Dako, Weybridge, UK) or goat antihuman IgG chain-specific antiserum (Sigma). After incubation of culture supernatants for two hours, alkaline phosphatase–conjugated goat antihuman antibodies specific for IgM or IgG (Sigma) were added for two hours. For visualization the substrate *p*-nitrophenyl phosphate (Sigma Phosphatase Substrate Tablets) was used. For determination of the monoclonality of the Ig produced, alkaline phosphatase–conjugated goat antihuman κ or λ light chain antibodies were used as the second step. All samples were tested for IgM and IgG production. The optical density of individual wells was determined with a Multiscan MC (Titertek, Flow Lab).

DNA and RNA incorporation assays. The cultures were pulsed with 1 µCi/well of ^{3}H-uridine or ^{3}H-thymidine (Amersham International, Amersham, UK) for one and six hours, respectively. The cells were harvested with a Multimash 2000 (Dynatech), and incorporation of ^{3}H-uridine into RNA and ^{3}H-thymidine into DNA was

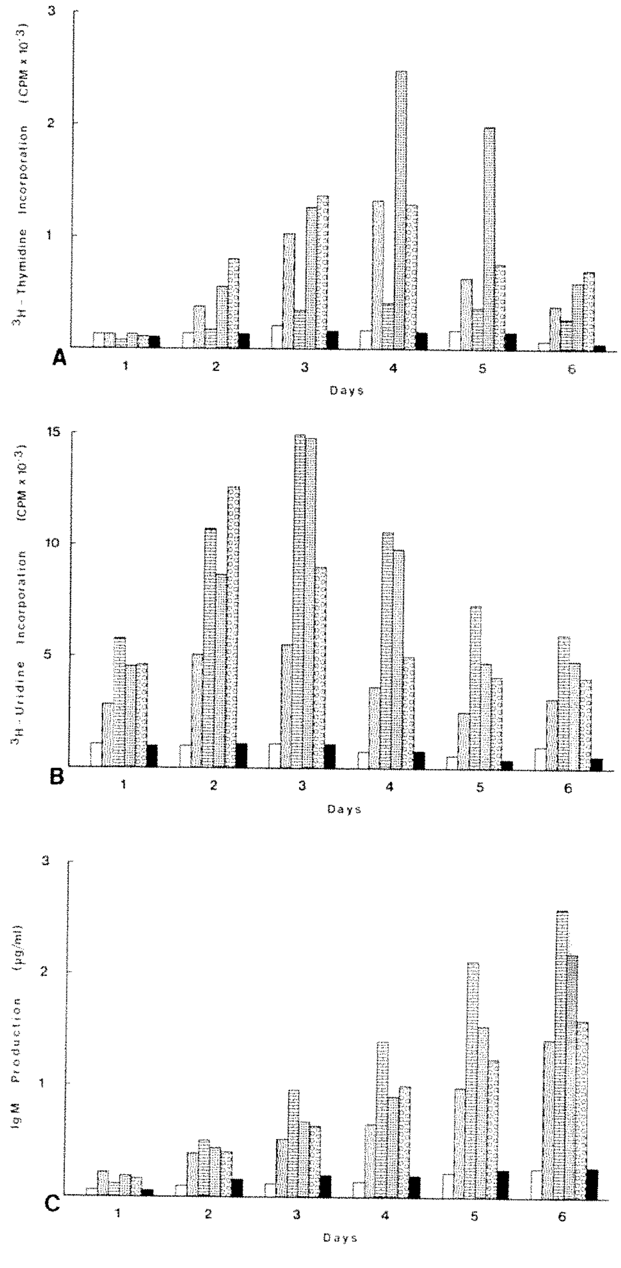

Fig 2. Response of B-CLL cells to continuous incubation with medium alone, TPA, A23187, and TPA plus A23187 in various concentrations measured at 24-hour intervals over six days. (A) ^{3}H-thymidine incorporation (patients 1 to 13). Uptake of ^{3}H-thymidine was significantly greater in the double-treated cultures than in the cultures treated with TPA or A23187 alone on days 3 and 4 (P = .025). (B) ^{3}H-uridine incorporation (patients 1 to 13). Uptake of ^{3}H-uridine was significantly greater in the double-treated cultures than in the cultures treated with TPA or A23187 alone on days 2 and 3 (P = .007). (C) IgM production (patient nos. 1 to 3 and 5 to 13). IgM production of the double-treated cells was significantly greater than in cultures treated with TPA or A23187 alone on days 4, 5, and 6 (P < .0001). □, Control; ▨, TPA; ▦, TPA + 0.7 µM A23187; ▤, TPA + 0.5 µM A23187; ▧, TPA + 0.3 µM A23187; ■, 0.5 µM A23187.

measured by standard liquid scintillation counting (LKB 1210 UltroBeta, Bromma, Sweden).

Statistical analysis. Comparisons between treatment groups were made by using paired *t* testing.

RESULTS

Cell populations. After depletion of T cells and adherent cells (monocytes, macrophages), the leukemia samples analyzed contained a neoplastic cell population of ≥98%. Less than 1% of the cells were positive for Leu-4 or RFT-2 (pan-T) or for monclonal antibodies detecting myelomonocytic antigens (MCS-2, MY4, Leu-M3).

The cells of all patients had morphological features typical of B-CLL, B-PLL, and HCL. The surface marker phenotypes are shown in Table 2. Besides characteristic morphology, the cases of B-PLL and HCL were positive for FMC7, which is a marker associated with but not specific for these leukemias.[13]

Normal B cells were obtained from the peripheral blood of healthy adults. However, these blood-borne normal B cells do not represent the normal counterparts to B-CLL cells (and therefore any comparison of the results is limited). B-CLL cells express the pan-T cell marker CD5, which is detected on <2% of normal peripheral blood B cells.[14] The T cell– and monocyte-depleted normal populations contained 28% to 42% cells stained by RFB7 (CD20).

Morphological changes. After 72 to 96 hours incubation, control B-CLL cells and A23187-treated cells were round, small, and nonadherent. TPA-exposed cells were either adherent to the surface of the culture dish with cytoplasmic elongations or clustered in densely packed, large clumps. Cells treated with TPA plus A23187 formed small clumps or aggregates that were nonadherent. B-PLL cultures showed similar growth changes.

HCL cells treated with either TPA alone or with the combination of TPA and calcium ionophore displayed prominent changes in their growth pattern as described elsewhere for TPA treatment of HCL[2]: the cells became strongly adherent and developed very long, thin, branched, cytoplasmic extensions. These cells could not be detached by vigorous shaking or pipetting with culture medium but required two cycles of incubation with trypsin solution.

On light microscopy, control B-CLL cells and A23187-exposed cells remained round with slightly increased cytoplasm. TPA-treated cells became larger, irregularly shaped, often with cytoplasmic vacuolization; however, the cytoplasm remained clear (Fig 1). In contrast, cells treated with the double stimulus enlarged but remained round to oval; the nuclei increased in size and moved to an eccentric position; the chromatin became finer, more evenly dispersed with nucleoli apparent; and the cytoplasm was deeply basophilic with a clear, "Hof"-like perinuclear zone. These plasmacytoid features were most pronounced in cells exposed to both TPA and 0.5 µmol/L A23187.

^{3}H-thymidine incorporation. In B-CLL cells, the double stimulus with TPA and A23187 increased the uptake of ^{3}H-thymidine into DNA significantly over the ^{3}H-thymidine incorporation found in cells treated with TPA alone ($P = .025$) (Fig 2A, Table 3). Maximal stimulation of proliferation occurred at 0.5 µmol/L A23187 plus TPA and peaked at day 4. At the highest ionophore concentration used, the response was less pronounced.

Both cases of B-PLL showed a severalfold higher incorporation of ^{3}H-thymidine (Table 3). In addition, the maximum response of B-PLL cells was at day 3 as opposed to day 4 in most B-CLL samples. The HCL cells were not responsive. In patient nos. 1 and 4 the response to the double stimulus did not surpass that to TPA alone; the cells from patient no. 3 could not be triggered to incorporate ^{3}H-thymidine (Table 4).

^{3}H-uridine incorporation. In the experiments summarized in Fig 2B, B-CLL cells were stimulated with either TPA or A23187 alone or TPA in combination with varying doses of A23187. The combined agents led to ^{3}H-uridine incorporation into RNA that was significantly increased over the activation with one of the inducers alone ($P = .007$). The highest peak was seen at day 3 with 0.7 µmol/L A23187 plus TPA.

Although the response of cells from patient no. 15 (B-PLL) was of about the same magnitude as seen in the B-CLL cultures, ^{3}H-uridine incorporation of cultures from patient no. 14 (B-PLL) was about four- to sixfold higher (Table 5). In both cases of B-PLL, the maximum response in terms of ^{3}H-uridine uptake occurred on day 2, whereas it was uniformly on day 3 in B-CLL cells. HCL cells did not incorporate significant amounts of ^{3}H-uridine (Table 5).

Ig production. The effect of the combined action of TPA and A23187 on Ig production was assessed in an ELISA

Table 3. Comparison of the Maximal ^{3}H-Thymidine Incorporation by B-CLL, B-PLL, and HCL Cells at Days 3 or 4 of Treatment With TPA, A23187, and TPA Plus A23187

Treatment	B-CLL (Patients 1-13)	B-PLL (Patient 14)	B-PLL (Patient 15)	HCL (Patient 16)	Normal B Cells (n = 3)
None (control)	177 ± 154*	229†	486†	473†	242 ± 94‡
TPA (2 × 10^{-8} mol/L)	1,318 ± 1,207	13,828	45,726	328	384 ± 240
TPA + 0.7 µmol/L A23187	413 ± 381	50,473	54,762	178	nd
TPA + 0.5 µmol/L A23187	2,478 ± 4,159	63,840	87,693	256	481 ± 304
TPA + 0.3 µmol/L A23187	1,288 ± 1,397	66,354	51,764	143	nd
A23187 (0.5 µmol/L)	159 ± 100	2,408	329	289	293 ± 164

Malignant cells were cultured at 4 × 10^6 cells per well and normal B cell populations at 0.5 × 10^5 cells per well.

Abbreviation: nd, not done.

*Counts per minute (mean ± SD from 13 patients).

†Counts per minute (mean from triplicate wells).

‡Counts per minute (mean ± SD from three donors).

Table 4. Response of Individual B-CLL, B-PLL and HCL Cases to Treatment With the Double Stimulus TPA Plus Calcium Ionophore

Patient No.	Diagnosis	Ig Production	^{3}H-Uridine Incorporation	^{3}H-Thymidine Incorporation
1	B-CLL	+*	+	−†
2	B-CLL	+	+	+
3	B-CLL	−‡	+	−‡
4	B-CLL	−†	−†	−†
5	B-CLL	+	+	+
6	B-CLL	+	+	+
7	B-CLL	+	+	+
8	B-CLL	+	+	+
9	B-CLL	+	+	+
10	B-CLL	+	+	+
11	B-CLL	+	+	+
12	B-CLL	−†	+	+
13	B-CLL	+	+	+
14	B-PLL	+	+	+
15	B-PLL	+	+	+
16	HCL	−‡	−‡	−‡

Patient no. 4 had an IgG isotype.

*Response to double stimulus greater than that to TPA alone.

†Response to double stimulus not greater than that to TPA alone.

‡No response to double or single stimulus.

assay. TPA induced a clear increase in Ig production in B-CLL cells (Fig 2C). This increase was not found in control or in A23187-treated cultures. The combination of the two agents induced the production of significantly higher amounts ($P < .0001$) of Ig than the exposure to TPA alone. The double stimulation with TPA plus 0.7 μmol/L A23187 was the most effective.

For all cases, IgM, IgG, and light chain production was measured. The response to TPA with or without calcium ionophore was monotypic in light and heavy chains and corresponded to the Ig light and heavy chains found by surface staining. In none of the cultures with IgM-positive cells was IgG or an increase thereof detected at levels above the sensitivity of the assay.

The B-PLL cultures showed a similar pattern of response in Ig production (Table 6). The HCL cells could not be induced to increase their Ig production (Table 6). In cultures from patient no. 4 (IgG isotype), TPA caused the cells to produce IgG; however, the response to the double stimulus was not higher than that to TPA alone (Table 4).

DISCUSSION

Recent studies observed a strong synergistic action of phorbol ester and calcium ionophores on the cell proliferation of normal human and murine B cells.[10,11,15,16] It was found that proliferation, cell size, and expression of the activation markers Tac, transferrin receptor, and HLA-DR of normal B cells induced by TPA was greatly enhanced by A23187.[10]

In a preliminary congress report on a small series of patients, Clevers et al have described that A23187 and phorbol esters act synergistically in the activation of B-CLL cells.[17] However, due to the lack of methodological, clinical, and immunophenotype data and the absence of a detailed presentation of the results, their report and the work presented here cannot be compared directly.

The present study demonstrates clearly that double stimulation of B-CLL cells with TPA and A23187 has significantly stronger effects than either reagent alone. The phenotypic changes in B-CLL cells induced by TPA have been described previously.[18-22] However, although the calcium ionophores used alone trigger cell activation in normal B and T cells,[4,5,23] we and others[7] found that A23187 alone is not effective in B-CLL cells. This discordance may be a consequence of the differentiation blockage of the malignant cells or may occur because T-depleted cell populations from normal donors have a higher percentage of contaminating T cells that are highly responsive to calcium ionophores. In preliminary experiments comparing B-CLL samples before and after T cell depletion we found that as few as 5% to 10% contaminating T cells altered these results significantly. It is therefore essential to purify the malignant B cell population and to verify the number of T cells in the resulting samples.

Higher concentrations of A23187 have been reported to inhibit DNA synthesis.[24,25] The range of useful doses of A23187 was very narrow in normal[25] and B-CLL cells.[7] In our system, the doses that synergized optimally with the phorbol ester to promote differentiation were in the narrow range of 0.3 to 0.7 μmol/L.

Interestingly, cell cycle progression studies on normal quiescent tonsillar B cells indicated that the combination of TPA and calcium ionophore stimulated essentially all cells,

Table 5. Comparison of the Maximal ^{3}H-Uridine Incorporation by B-CLL, B-PLL, and HCL Cells at Days 2 or 3 of Treatment With TPA, A23187, and TPA Plus A23187

Treatment	B-CLL (Patient nos. 1-13)	B-PLL (Patient no. 14)	B-PLL (Patient no. 15)	HCL (Patient no. 16)	Normal B Cells ($n = 4$)
None (control)	1,094 ± 1,158*	2,202†	303†	277†	323 ± 54‡
TPA (2 × 10^{-8} mol/L)	5,566 ± 4,866	34,373	1,303	694	806 ± 419
TPA + 0.7 μmol/L A23187	14,942 ± 13,031	48,185	6,122	623	nd
TPA + 0.5 μmol/L A23187	14,797 ± 16,790	54,006	17,420	720	1,686 ± 1,006
TPA + 0.3 μmol/L A23187	9,217 ± 7,128	55,210	8,312	394	nd
A23187 (0.5 μmol/L)	1,138 ± 1,192	1,832	1,017	298	648 ± 71

Malignant cells were cultured at 4 × 10^5 cells per well and normal B cell populations at 0.5 × 10^5 cells per well.

Abbreviation: nd, not done.

*Counts per minute (mean ± SD from 13 patients).

†Counts per minute (mean from triplicate wells).

‡Counts per minute (mean ± SD from four donors).

Table 6. Comparison of Ig Production by Treated B-CLL, B-PLL, and HCL Cells at Day 6 of Treatment With TPA, A23187, and TPA Plus A23187

Treatment	B-CLL (Patient nos. 1-3, 5-13)*	B-PLL (Patient no. 14)	B-PLL (Patient no. 15)	HCL (Patient no. 16)	Normal B Cells (n = 4)
None (control)	0.21 ± 0.17†	0.20	0.08	<0.05‡	0.21 ± 0.05§
TPA (2 × 10^{-8} mol/L)	1.42 ± 1.38	0.93	0.49	<0.05	0.20 ± 0.07
TPA + 0.7 μmol/L A23187	2.60 ± 0.87	2.02	2.04	<0.05	nd
TPA + 0.5 μmol/L A23187	2.23 ± 0.94	1.64	1.53	<0.05	0.28 ± 0.10
TPA + 0.3 μmol/L A23187	1.60 ± 0.68	1.38	0.86	<0.05	nd
A23187 (0.5 μmol/L)	0.27 ± 0.24	0.23	0.12	<0.05	0.12 ± 0.04

Malignant cells were cultured at 4 × 10^5 cells per well and normal B cell populations at 0.5 × 10^5 cells per well.

Abbreviation: nd, not done.

*Patient no. 4 was of the IgG isotype.

†Micrograms per milliliter (mean ± SD from 12 patients).

‡Below the sensitivity of the ELISA assay.

§Micrograms per milliliter (mean ± SD from four donors).

whereas TPA alone triggered only a proportion of cells.[26] We confirm these findings by observing that the majority of the cells per sample changed their morphology to plasmacytoid variants upon exposure to the double stimulus. Similarly, the observed increase in thymidine and uridine uptake probably represents stimulation of a higher proportion of cells by TPA and calcium ionophore together than either in isolation. However, it remains possible that nucleotide uptake is enhanced due to increased stimulation of a minority subpopulation of cells.

Using normal tonsillar, spleen, and peripheral blood B cells, Clevers et al[10] demonstrated that TPA and A23187 did not induce differentiation of these cells to Ig production. These results stand in contrast to our experiments where the synergy of the two stimuli clearly caused a strong increase of Ig production in B-CLL cells. However, these authors noted that doubly stimulated normal B cells subsequently exposed to B cell differentiation factor (BCDF) produced large amounts of Ig. This suggests that TPA plus calcium ionophore provided increased responsiveness of normal B cells to differentiation factors.[10] It is not clear why the malignant cells treated with the double stimulus responded promptly by secreting significant amounts of Ig with no further requirement for BCDF. One possible explanation is the autocrine secretion of differentiation-inducing factor(s),[27] and this possibility is being explored by using several growth/differentiation factors.

The morphology of PLL cells and CLL cells in prolymphocytoid transformation resembles that described for activated normal B cells,[28] and these B-PLL cells respond more strongly than B-CLL cells to the double stimulus. On the other hand, HCL cells did not respond to phorbol ester and calcium ionophore in terms of differentiation toward plasma cells. Despite the fact that HCL cells have been shown to be of B cell derivation[29] and have even been classified by immunologic means as "preplasma cells,"[30] they did not develop plasmacytoid features typical of secreting B cells either during treatment with TPA alone[2] or during exposure to the double stimulus. However, certain morphological changes were found in treated HCL cells, and the discrepancy between morphological alterations of HCL cells and their unresponsiveness in other respects is of interest. The lack of responsiveness of HCL cells might be the result of a possible dependence on growth/differentiation factor(s). Further studies are required to address this question.

In summary, the results presented indicate that (a) phorbol ester and calcium ionophore act synergistically in B-CLL in inducing proliferation and differentiation and (b) the capacity of B-CLL cells to differentiate in response to signals of the second messenger pathway is intact.

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Limiting-Dilution Analysis for the Determination of Leukemic Cell Frequencies After Bone Marrow Decontamination With Mafosfamide or Merocyanine 540

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To simulate a leukemia remission marrow, cell suspensions of normal human bone marrow were mixed with human acute lymphoblastic or myelogenous leukemic cells of the CCRF-SF, Nalm-6, and K-562 lines. The cell mixtures were incubated in vitro with mafosfamide (AZ) or with the photoreactive dye merocyanine 540 (MC-540). A quantity of 10^4 cells of the treated suspensions was dispensed into microculture plates, and graded cell numbers of the line used to contaminate the normal marrow were added. Limiting-dilution analysis was used to estimate the frequency of leukemia cells persisting after treatment with the decontaminating agents. Treatment with AZ or MC-540 produced a total elimination (ie, 6 logs or 5.3 logs respectively) of B cell acute leukemia cells (CCRF-SB), whereas nearly 1.7 logs and 2 logs of K-562 acute myelogenous blasts were still present in the cell mixtures after treatment with MC-540 and AZ, respectively. Treatment of the Nalm-6–contaminated cell mixtures with AZ resulted in 100% elimination of clonogenic cells, whereas nearly 80% decontamination was obtained with MC-540. Our results suggest that treatment with AZ could be an effective method of eliminating clonogenic tumor cells from human bone marrow. MC-540, shown by previous studies to spare sufficient pluripotential stem cells to ensure hemopoietic reconstitution in the murine model and in clinical application, has comparable effects and merits trials for possible clinical use in autologous bone marrow transplantation.

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AUTOLOGOUS BONE MARROW transplantation (ABMT) is now being used as an alternative transplant approach for patients in remission of acute leukemia who lack an HLA-identical donor. The major stumbling block in ABMT still remains the high relapse rate as compared with allogeneic marrow transplantation,[1] evidently due to occult leukemic cells that contaminate the remission marrow. Several methods for eliminating these cells from autologous marrow have been proposed, but the efficacy of the purging procedures has yet to be established. Although the advantage of marrow purging is uncertain in acute nonlymphocytic leukemia (ANLL) treated by autotransplantation in the first complete remission (CR), there are some indications that cleansing of the marrow is linked with improved survival in autograft recipients in the second CR.[1] Despite the preliminary nature of these data and the need for more prolonged follow-up, in vitro treatment of the autologous marrow harvest to eliminate or at least reduce leukemic cells would seem a desirable goal.[2]

4-hydroperoxycyclophosphamide (4HC) and 4-hydroxazaphosphorine or mafosfamide (AZ), both congeners of cyclophosphamide (CY), are the most commonly used drugs for chemical purging in ABMT. Evidence from animal models that 4HC and AZ may purge marrow of clonogenic cells[3,4] while sparing the pluripotential stem cells[5] has been presented previously. Furthermore, 4HC- or AZ-treated human marrow cell suspensions are able to reconstitute the hematopoietic system after ablative therapy despite a marked reduction in detectable progenitor cells.[2,6-10]

The photoreactive dye merocyanine-540 (MC-540) binds to fluid like domains in the outer leaflet of the lipid bilayer of intact cell membranes.[11-14] Electrically excitable cells and some immature hematopoietic precursor cells show exceptional permeability to MC-540.[15]

Photoexcitation of membrane-bound dye with light of suitable wavelength causes increased dye uptake, impairment of the normal permeability properties of the plasma membrane, and eventually, cell death.[15,16] Sieber et al[17-20] have studied the sensitivity to MC-540–mediated photolysis of hematopoietic precursors and pluripotential stem cells as compared with the lymphocytic leukemia cell line L1210. Their findings of a much greater reduction in the L1210 cell number suggest that this dye might serve as a cleansing agent for ABMT.

Although marrow cleansing offers an interesting approach because the aforementioned research and clinical trials have shown that this procedure is not harmful to pluripotential stem cells and not hazardous, the real efficacy of this procedure in terms of residual clonogenic cell removal still needs to be determined. Several assays have been developed in an attempt to solve this problem.[21-26]

This study was designed to test the ability of AZ or MC-540 to eliminate clonogenic leukemic cells from a mixture of leukemic and normal human bone marrow mononuclear cells (HBMMC). For this purpose, a highly sensitive in vitro clonogenic assay was developed by using established human leukemia cell lines. This assay, which enabled enumeration of residual leukemia cells, was sufficiently sensitive to detect as much as a 6-log reduction in leukemia cells that contaminate human bone marrow.

MATERIALS AND METHODS

Preparation of bone marrow suspensions. Bone marrow specimens were aspirated into heparinized syringes from the posterior iliac crests of healthy volunteers after obtaining their informed consent. The mononuclear marrow cell line suspensions were prepared as described previously.[27]

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Leukemia cell lines. The following established cell lines were used: pre-B cell line Nalm-6; B cell line CCRF-SB, and the myeloid-monocytic line K-562. All cell lines were kindly supplied to us by Dr Hansen, Fred Hutchinson Cancer Research Center, Seattle. The cell lines were maintained in RPMI 1640 medium with 15% fetal calf serum (FCS), 1% penicillin-streptomycin antibiotics (10,000 U), 1% L-glutamine, and 1% minimal essential medium (MEM) sodium pyruvate solution and were kept at 37°C in a fully humidified incubator with 5% CO_2. The cells were maintained in exponential growth by keeping the cell concentration in a range from 0.5 to 1.0×10^6 cells/mL. The viability of all cell lines was $\geq 90\%$ as judged by trypan blue dye exclusion.

Purging agents. AZ (Asta Werke, Bielefeld, FRG) was diluted with TC 199 to a concentration of 10 μg/mL immediately before use.

MC-540 (Eastman Kodak Co, Rochester, NY) was diluted from a freshly prepared stock solution of 1 mg/mL in 50% (vol/vol) water-ethanol to a final concentration of 15 μg/mL.

Purification with AZ. To simulate remission bone marrows from leukemia patients, 10^6 leukemia cells of the CCRF-SB, Nalm-6, or K-562 cell lines were mixed with a 19-fold excess of HBMMC; the suspensions were adjusted to 20×10^6 cells/mL with TC 199 medium, placed in 12-mL plastic tubes, and incubated for 30 minutes at 37°C in a water bath with 50 or 100 μg/mL of AZ. After incubation, the cell suspensions were placed in ice for five minutes to stop the reaction and then washed twice in RPMI 1640.

Photosensitization with MC-540. A quantity of 2×10^5 leukemia cells of the CCRF-SB or K-562 lines were mixed with a 19-fold excess of HBMMC; the suspension was adjusted to 4×10^6 cells/mL with MEM α medium (GIBCO, Grand Island, NY), containing 15% FCS (Seromed, FRG) and placed in Petri dishes, and 15 μg of MC-540 dissolved in 50% ethanol was added. Clear polystyrene tissue culture dishes (60×15 mm) containing the cell suspension were placed in the center of a rectangular frame (58×40 cm) fitted with six daylight fluorescent tubes (Phillips, TL M 20W/133 RS, 56 cm long), three above the dishes and three below, and illuminated for 45 or 90 minutes. Illumination at each side of the sample (at 20 cm from the light source) was 0.0007 W/cm^2.

The dishes were shaken manually every five minutes to keep the cells in suspension. After light exposure, the cell suspensions were washed twice in MEM α medium containing 5% FCS.

Limiting-dilution Assay. After incubation with AZ or MC-540, 10^4 cells from those treated were suspended in 100 μL RPMI 1640 and plated in microculture plates (Nunc, Roskilde, Denmark).

Graded cell numbers of the same line used to contaminate the HBMMC were suspended in 100 μL RPMI 1640 containing 15% pooled human serum and added to the treated cell feeders and incubated for 13 days. Refeeding of the cultures with fresh medium was carried out every third day.

Twelve hours before harvesting, the cells were pulse-labeled with 1 μC; ^{3}H-thymidine. The cells were then harvested by means of a Skatron apparatus (Lierbyen, Norway), and incorporated thymidine was quantitated by scintillation counting.

Statistical analysis. Clonogenic cell frequency was determined by the maximum likelihood (ML) method from the plot of the Poisson distribution relationship between the cell number seeded per well and the percentage of negative wells.

The ML equation was obtained by modifying the Taswell method[28]; ML regression analysis, x-intercept values, and confidence limits on the population regression line for prediction of y at a given value of x were obtained by using the MINUIT computer program (CERN Library, Geneva) in a VAX-750 computer.

RESULTS

For this study, a limiting-dilution (LD) analysis was adopted to evaluate the frequency of leukemic cells of the CCRF-SB, Nalm-6, and K-562 lines surviving in an HBMMC mixture after treatment with AZ or MC-540.

The mean ^{3}H-thymidine uptake in the wells in which no leukemia cells were added (ie, containing only 10^4 cleansed HBMM cells) was determined (Fig 1). Only wells that exceeded the mean of the control wells by at least 3 SD were scored as positive (responders). The number of responders increased progressively with the number of blasts added (Fig

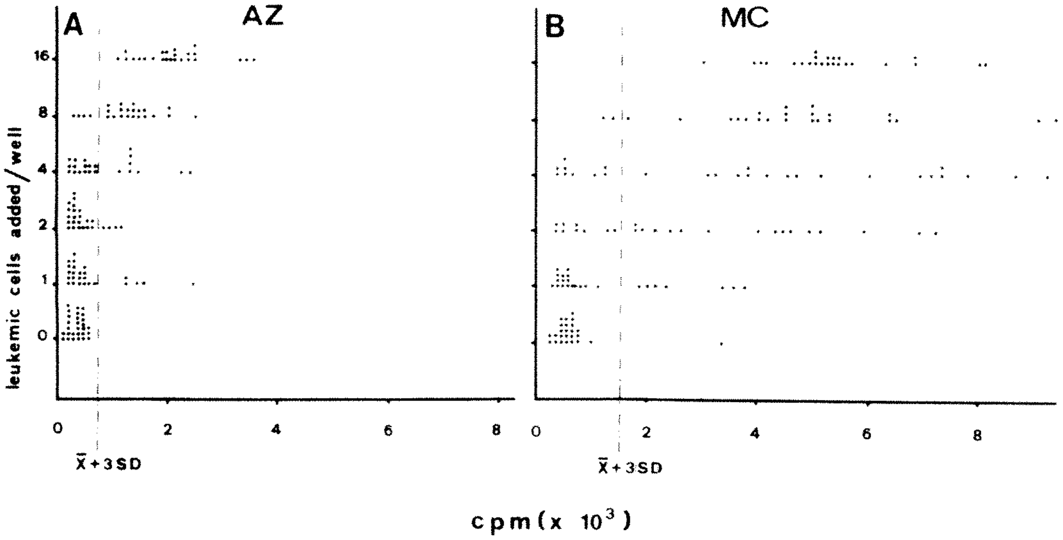

Fig 1. Distribution of ^{3}H-thymidine uptake in an LD assay for residual CCRF-SB lymphoblasts. Groups of 24 microwells containing suspensions of 10^4 mixed cells treated with AZ (lefthand panel) or MC-540 (right-hand panel) were seeded with graded numbers of CCRF-SB cells per well (see Materials and Methods) and incubated for 13 days. The cell proliferation rate was assayed by ^{3}H-thymidine uptake. The dotted line represents 3 SD above the mean ^{3}H-thymidine uptake of the nonresponding microcultures with no added CCRF-SB clonogenic cells.

1). Figures 2 and 3 represent the data obtained from LD assays of cleansed HBMM and leukemic cell mixtures in which the equations of the line of best fit were derived by ML methods.

The cloning efficiencies of CCRF-SB, K-562, and Nalm-6 cells in this experiment, calculated as the slope of the regression line when the ordinate is the natural log of the percent nonresponders (Fig 2), were 22%, 44%, and 26% respectively.

The estimate of the number of leukemic cells still present in the cell mixtures after cleansing is represented by the x-intercept of the regression line. In the experiment shown in Fig 2, complete decontamination of CCRF-SB and Nalm-6 cells (panels A and C) in each well containing 10^4 mafosfamide-purged mixture cells was obtained, and 0.08 K-562 leukemic cells (Panel B) survived after treatment.

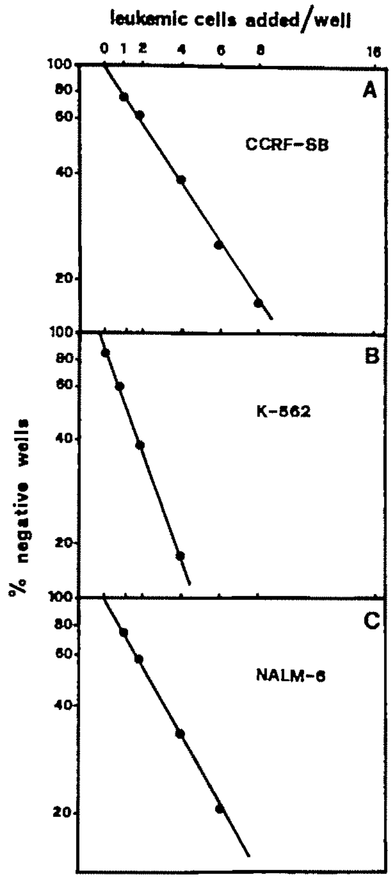

Fig 2. LD analysis of mafosfamide-treated bone marrow mononuclear cells previously contaminated with 5% of one of the following clonogenic cell lines: CCRF-SB lymphoblasts (A), myelogenous K-562 cells (B), and pre-B Nalm-6 cells (C). The percentage of nonresponding wells is plotted against the number of clonogenic cells added to each well containing 10^4 purged mixture cells. The wells were scored as positive (responders) or negative (nonresponders) according to whether the ^{3}H-thymidine uptake was greater or less than the threshold 3 SD above the mean of the nonresponding microcultures with no added clonogenic cells. The lines of best fit, as determined by ML regression analysis, x-intercept values, and confidence limits on the population regression line, were originated by the MINUIT computer program in a VAX-750 computer.

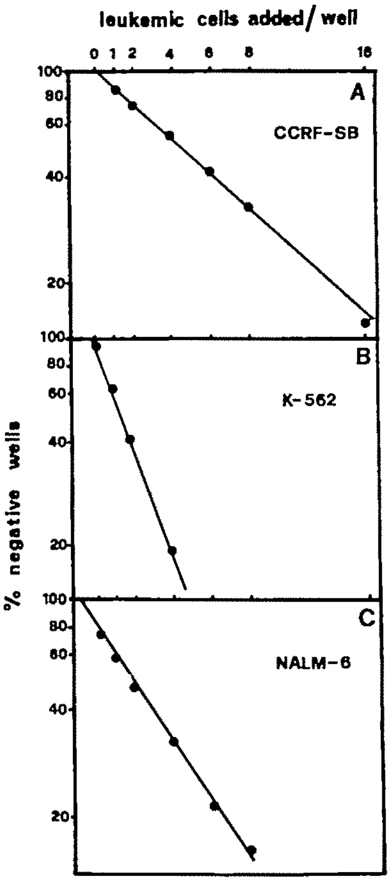

Fig 3. Results obtained from LD analysis of MC-540-treated bone marrow mononuclear cells; details are as in Fig 2.

The 95% confidence intervals (95% CI) for the value of the x-intercept of this experiment, calculated as described by Cox and Hinkley,[29] were −0.71 to 0, −0.28 to 0, and −0.39 to 0 for the three leukemic lines respectively. Thus, there was <5% probability that the frequency of leukemic cells would be greater than $1/10^4$ cleansed mixture cells. It should be noted that the threshold sensitivity of this assay was about one clonogenic cell per 10^4 HBMM cells (Figs 2 and 3).

Similar results were obtained in two replicate experiments in which a complete clearing of all leukemic cells was obtained, with the exception of 0.16 residual K-562 blasts in one single experiment. In all these replicate studies, the 95% CI indicated that there was always a 5% probability that the frequency of residual blasts would be $>1/10^4$ purged mixture cells.

Figure 3 shows the results obtained by purging the cell mixtures with MC-540. With this cleansing agent, the calculated residual clonogenic cells in each well were 0 CCRF-SB cells (95% CI, <0.38, panel A) and 0.11 K-562 cells (95% CI <0.53; panel B). Pre-B Nalm-6 cells proved less responsive to MC-540 in that 1.78 residual leukemic cells (95% CI, <4.74) were found after photosensitization. The cloning efficiencies of leukemic cells in this experiment as judged by the ML regression line were 13.4% (CCRF-SB), 40.3% (K-562), and 8.7% (Nalm-6).

These results were confirmed in two replicate experiments. The B cell lymphoblasts were eliminated completely by the MC-540 treatment; however, 0.023 to 0.18 clonogenic cells were present after purging cell mixtures contaminated with the K-562 cells. Again, the 95% CI confirmed that there was a 5% probability of the residual blasts being present at a frequency greater than $1/10^4$ purged mixture cells.

The effectiveness of both AZ and MC-540 proved to depend directly on the dose level (AZ) and the light exposure time (MC-540) (Fig 4). Incubation of the cell mixture containing CCRF-SB clonogenic cells with 50 μg/mL of AZ caused decontamination of 3.2 logs; hence about 620 clonogenic cells persisted after purging. The cloning efficiency in this experiment was 8.6%. By increasing the dose of AZ to the usual level of 100 μg/mL, complete clearing of CCRF-SB lymphoblasts was obtained, and the cloning efficiency was increased to 16.5% (Fig 4, left-hand panel).

With an exposure time of only 45 minutes, MC-540 was able to decrease the CCRF-SB lymphoblast frequency in the cell mixture by 3.7 logs. About 40 clonogenic cells were present after purging, and the ML regression line showed a cloning efficiency of 2.3%. By contrast, an exposure time of 90 minutes not only caused complete clearing of the lymphoblasts but also increased the cloning efficiency to 7.3%. By lengthening the exposure time to 120 minutes, we could not detect a higher degree of decontamination; however, the cloning efficiency obtained in this case was 26.1% (Fig 4, right-hand panel).

DISCUSSION

Removal of occult malignant cells may be of critical importance for improving the efficacy of ABMT in acute leukemia.[1,7] A major complication in this procedure is the impossibility of evaluating the effects of in vitro treatment both on cells responsible for engraftment and on clonogenic cell removal.

Evidence obtained in experimental models that 4-HC, AZ, or MC-540 does not impair the ability of marrow cell suspensions to restore hematopoiesis has already been presented.[3,5,18]

A test for pluripotential stem cell survival in humans is not currently available. However, a body of evidence from both experimental and clinical studies indicates that a sufficient number of cells responsible for hematopoietic reconstitution survive exposure to either CY derivatives or MC-540. Indirect confirmation thereof was obtained in two in vitro studies. Normal human marrow cell suspensions treated with 4-HC and then seeded in a long-term marrow culture system, although completely devoid of detectable progenitor cells, showed recovery of CFU-GM.[30] A similar study in which marrow from leukemic patients in CR was incubated with AZ obtained comparable results.[8] Both studies tested a variety of drug dose levels including those commonly used in clinical ABMT and clearly indicated that purged marrow suspensions retain their self-regenerating ability.

The effect of both 4-HC and AZ on normal hematopoiesis was evaluated by Gordon et al[31] and Deglantoni et al[32] by means of a new colony assay[33] designed to detect very immature blast progenitor cells. Both these studies showed that in vitro treatment with either drug did not impair the formation of blast colonies containing very primitive cells capable of self-renewal and differentiation.

An indirect but persuasive demonstration that ex vivo purging with CY derivatives is not harmful to pluripotential stem cells may be inferred from the prompt restoration of hematopoiesis after reinfusion of purged marrow suspensions following myeloablative treatment.[2,7-10] In these reports the

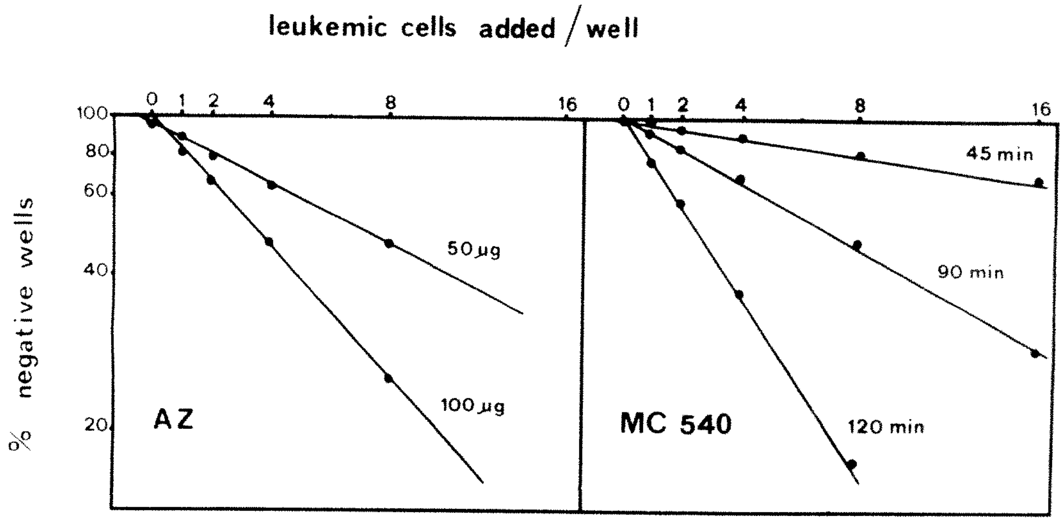

Fig 4. Semilog plots of the proportion of nonresponding microcultures for each dose of clonogenic cells added to wells containing suspensions of 10^4 cleansed cells which were previously contaminated with 5% CCRF-SB lymphoblasts. Results were obtained from LD analysis of cell mixtures decontaminated with AZ or MC-540. Lefthand panel, 19 × 10^6 HBMM cells contaminated with 10^6 CCRF-SB lymphoblasts were treated with 50 or 100 μg/mL of AZ. Righthand panel, 3.8 × 10^6 HBMM cells contaminated with 2 × 10^5 CCRF-SB lymphoblasts were incubated with 15 μg/mL MC-540 under fluorescent lights for 45, 90, and 120 minutes.

median time of hematopoietic recovery was comparable to that obtained in ABMT with untreated marrow.

In the first clinical application of ABMT purged with MC-540, the photoreactive dye showed that it did not compromise the graft's ability to reconstitute the hematopoietic system.[34]

The other major concern in ex vivo marrow cleansing is to quantitate the number of clonogenic cells present in the remission marrow and to estimate those removed by the cleansing procedures.

Unfortunately there is no means available for detecting minimal residual disease in remission marrow. However, by using leukemia cell lines it is possible to develop clonogenic assays that measure the elimination of malignant cells from an excess of normal HBMMC. Although these model systems may differ somewhat from the clinical situation, such in vitro simulation experiments permit evaluation of the efficiency of the drug used in terms of leukemic cell removal.

The LD assay has been used extensively to study the response of antigen-reactive cells both in animal models[35,36] and in humans.[37-39] To meet our requirements, we have adapted an LD assay originally described by Moretta et al[40] and modified by Martin and Hansen.[41]

In all the experiments performed, the ML regression lines were consistent with the single-hit model (Figs 2 and 3). Assuming that the test mixture cell suspensions are homogeneous, the leukemic cells sampled into each microwell will occur randomly according to the Poisson probability distribution. In a single-hit model, it is not possible to determine whether a positive response is due to one or more cells. However, no response occurs in the absence of cells.

Therefore, the zero term of the Poisson equation will indicate that the proportion of nonresponders is a negative logarithmic function of the number of leukemic cells added to each well; hence in our study, it was possible to establish an equation for the line of best fit to calculate the leukemic cell frequency in each sample. The efficacy of this assay hinges on its ability to provide quantal discrimination between wells with cell growth (responders) and wells with no cell growth (nonresponders).[41] Definition of the latter is somewhat arbitrary because when total decontamination (100% purging) was not attained some leukemic cells were present in the wells after treatment even when none were added for the LD assay. However, in all our experiments, the outliers in ^{3}H-thymidine uptake were quite obvious (Fig 1B) and were disregarded when calculating the cutoff between negative and positive wells. In some cases, when there is a high number of residual leukemic cells or when microcultures are seeded with an excessive amount of leukemic cells, the increased proliferation rate of the leukemic cells, as measured by ^{3}H-thymidine incorporation, can give rise to false-negative results and hence an unexpectedly high proportion of negative wells. In our microtray assay system, although cultures are refed periodically, medium supply may become a limiting factor in the case of an excessive cell proliferation rate. It is possible to adjust the LD assay for different degrees of blast cell purging by changing the number of clonogenic cells seeded per well.

LD assay techniques usually involve the use of irradiated feeders to which increasing numbers of cells containing an unknown proportion of precursors are added. Hence, it follows that when no precursors are added to the irradiated feeder-containing wells 100% nonresponders will be obtained, and the regression line will necessarily include the origin. In our experiments, the feeders (ie, 10^4 purged HBMM cells) contained an unknown proportion of residual leukemia cells to which further successive known doses of blasts were added. Thus, one would not expect semilog plots of blast cells added and the proportion of nonresponders to necessarily include the origin, except in the case in which no residual blasts remained in the wells.

Should residual blasts persist after purging, the x-intercept that represents an estimate of the number of blast cells that would have to be removed from the feeders to obtain 100% nonresponders can be calculated from the ML regression line.

In our model system, after AZ or MC-540 treatment at the highest dose level tested, no acute leukemia CCRF-SB lymphoblasts were detectable in the cell mixtures. In the Nalm-6–contaminated mixtures, AZ obtained 100% decontamination, whereas the figure for MC-540 was 80%. In the mixtures containing K-562 cells, both agents yielded a result of 99.97% decontamination. Our data do not indicate whether it would be possible to obtain a higher degree of decontamination, either by increasing the dose level of AZ or by increasing both the dose level and the light exposure time of MC-540. Clinical experience has shown that 100 μg/mL of AZ is the maximum safe dose level for this drug, and further increases might be critical.[2] As far as MC-540 is concerned, further studies are needed to determine potential harmful effects on pluripotential stem cells at doses higher than those used in our model.

It is possible that occult leukemic cells contaminating a remission marrow are more difficult to eliminate than exponentially growing leukemic cells such as those used in our model system. It may be more difficult to obtain 99.97% decontamination in the clinical situation if the clonogenic leukemic cells are quiescent or slowly cycling. Thus the figure of 99.97% may represent an overestimate of the cleansing efficiency of both AZ and MC-540. Moreover, because the threshold limit for detection with this assay is about one per 500 clonogenic cells, it is evident that when complete decontamination (as calculated by our LD assay) is obtained as many as 3 to 4 logs of clonogenic cells could persist after purging, given the supposed initial leukemic cell population in the autologous harvest of between 10^6 and 2×10^7 cells.

It has been suggested that the susceptibility of tumor cells to alkylating agents may be due to a decrease in their aldehyde dehydrogenase content (compared with normal cells) rather than to their proliferative state.[42-44] However, actively proliferating cells are likely to prove more susceptible to alkylators than are dormant cells. Moreover, if ABMT is performed in the second or higher CR, residual malignant cells may have developed a relative resistance to alkylating agents. One potential advantage of using MC-540 is that this agent causes impairment of the normal permeability properties of the plasma membrane regardless of the proliferation rate of target cells. This suggests that this approach could effectively eliminate noncycling malignant cells as well.[16]

The viability of marrow treated with MC-540 is yet to be evaluated in a phase I clinical trial. However, our in vitro data suggest that chemical treatment of marrow with this photoreactive dye might provide a new approach for removing malignant cells, regardless of their cycling rate, without unduly damaging the pluripotential stem cells.

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Evidence for Peripheral Blood B Lymphocyte but Not T Lymphocyte Involvement in Multiple Myeloma

By James Berenson, Rex Wong, Kenneth Kim, Nathaniel Brown, and Alan Lichtenstein

Because there is controversy regarding whether subsets of peripheral blood lymphocytes (PBLs) are part of the malignant clone in patients with multiple myeloma, we studied this question by immunoglobulin and T cell receptor gene analysis. Southern blot analysis with antibody probes demonstrated clonal immunoglobulin gene rearrangements in PBLs of seven of nine patients that were identical to those seen in their marrow plasma cells. Circulating plasma cells were not detected in any of these patients. In contrast, no patient demonstrated clonally rearranged T cell receptor genes. In one sequentially studied patient, PBLs obtained at diagnosis when he had stage I (Durie-Salmon) contained only germline DNA, while analysis of PBLs at relapse (stage III) revealed a clonally rearranged band. These data confirm the notion that circulating lymphocytes in patients with myeloma are part of the malignant clone and, furthermore, these malignant cells are of B cell rather than T cell lineage.

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MULTIPLE MYELOMA is regarded as a disease of immunoglobulin-producing cells. Although the predominant cell of this marrow-based neoplasm has the morphological characteristics of a plasma cell,[1] data have accumulated that other lymphoid cell types are also part of the malignant process.[2-5] In patients, a significant number of peripheral blood lymphocytes (PBLs) bear the unique paraprotein idiotype found in the plasma cells. These data would suggest that an earlier cell in B cell differentiation is part of the malignant clone. Moreover, some workers have shown that idiotype-bearing T cells also circulate in the peripheral blood of these patients.[6]

On the other hand, King and Wells[7] have demonstrated that the cytophilic IgG idiotype may remain bound to Fc receptors on PBLs even after extensive washing and incubation. These investigators failed to show any evidence of PBLs with an endogenous surface idiotype when using a rosette assay. Recently, two other groups have also not found significant numbers of idiotype-bearing PBLs when using similar assays.[8,9] Thus, there is controversy regarding whether PBLs are part of the malignant clone in multiple myeloma and, furthermore, which lymphocyte subsets are involved in the disease.

To address this controversy, we have searched for antibody and T cell receptor (TCR) gene rearrangements to determine whether significant numbers of clonal malignant B and/or T lymphocytes exist in the peripheral blood of patients. Gene rearrangements can be used to accomplish this goal because clonally derived B or T cell populations have unique rearrangements of antibody or TCR genes, respectively. This DNA analysis avoids the problems with passively adsorbed antibody which has confounded previous studies.

MATERIALS AND METHODS

Patients. Nine patients with multiple myeloma as defined by the Myeloma Task Force were studied.[10] All patients underwent bone marrow examination, serum protein electrophoresis, immunoelectrophoresis, bone surveys, routine chemistries, CBCs, and 24-hour urine collection for determination of creatinine and protein. Patients were staged according to the Durie-Salmon classification.[11]

Isolation of peripheral blood lymphocytes, granulocytes and bone marrow plasma cells. Peripheral venous blood and bone marrow samples were obtained from all nine patients after informed consent and in accordance with the Human Subjects Review Boards of the Veteren's Administration Wadsworth and UCLA Medical Center. Peripheral blood was centrifuged to separate the cells from the serum. The cells were exposed to Histopaque (Pharmacia Fine Chemicals, Piscataway, NJ) 1077 and 1119 density gradient separation. This technique yields >98% pure granulocytes at the 1077/1119 interface, and these cells do not show antibody gene rearrangements in Southern blot analysis.[12] The mononuclear cells appear at the 1077/Hanks' balanced saline solution interface, and the monocytes are removed from this fraction by adherence to plastic dishes, which leaves highly purified PBLs (>98%). Bone marrow was exposed to Ficoll-Hypaque (Pharmacia) density gradient separation to yield enriched populations of plasma cells.

Southern blot analysis. Genomic DNA was prepared from PBLs, granulocytes, and bone marrow plasma cells on each patient according to the method of Perry et al.[13] The DNA was digested with restriction endonucleases *Bam*HI, *Eco*RI or *Hin*dIII (Bethesda Research Laboratories, MD). Ten micrograms of digested DNA was electrophoresed in 0.6% agarose gels at 1 to 2 V/cm for 42 hours and transferred to nylon membranes by the method of Southern.[14] Parallel lanes of similarly digested granulocyte, PBL, and plasma cell DNA samples from each patient were electrophoresed on the same agarose gel.

Recombinant probes for the human heavy chain immunoglobulin (Ig) gene segments J_H and $C_{\gamma 4}$ were donated by L. Hood et al (California Institute of Technology, Pasadena, CA) and were subcloned in pBR322. The J_H probe is a 3.2-kilobase pair (kb), human genomic fragment extending from JH2 to the *Hin*dIII site in the J_H-C_μ intervening sequence.[15] The $C_{\gamma 4}$ probe is a 2-kb genomic clone containing the complete sequence of $C_{\gamma 4}$; this probe hybridizes with all four C_γ-subclass gene fragments, which are reported to be ≥90%

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homologous.[16] The C_α probe is a 4.2-kb *Bam*HI-*Xho*I human genomic fragment that contains the $C_{\alpha 1}$ gene subcloned in pBR322 and was subcloned from a Charon 28 phage that was donated by J. Ravetch et al (Harvard University, Cambridge, MA).[17] Recombinant probes for the human light chain Ig gene segments C_λ and J_κ-C_κ were donated by P. Leder. The C_λ probe is a 5.0-kb *Eco*RI-*Hind*III human genomic fragment that is also subcloned in pBR322.[18] The J_κ-C_κ probe is a 2.5-kb *Eco*RI fragment in pBR325.[19] The TCR gene probe is a β-TCR cDNA clone called JUR-beta 2, subcloned in pBR322 and donated by Dr. T. Mak (University of Toronto).[20]

Probe DNAs were labeled with $[^{32}P]$ dCTP (deoxycytidine triphosphate) by nick translation.[14] Hybridization and washing were essentially those described by Wahl et al.[21] After washing, the hybridized blots were autoradiographed using Kodak XAR-5 film and intensifying screens at −70°C for four to 14 days.

RESULTS

All patients were male and ranged from 53 to 79 years of age. Six patients had IgGκ myeloma, two had IgAλ myeloma, and one had IgGλ myeloma (Table 1). Three patients were in stage IIA; three, IIIB; two, IIIA; and one, IA at diagnosis and IIIB at relapse (patient no. 6, Table 1). No patient had obvious circulating plasma cells on microscopic examination of their peripheral blood.

The results of Southern blot analysis with antibody and β-TCR gene probes are summarized in Table 1. Seven of nine patients showed clonal antibody gene rearrangements in PBLs identical to those seen in their respective marrow plasma cells. In all six patients with IgGκ myeloma, this was demonstrated by identification of rearranged light-chain genes and/or heavy-chain genes. In one of two IgA patients, a rearranged light-chain band was detected, whereas only germline DNA was found in the other. In the one patient with IgGλ myeloma, hybridization with the J_H probe only revealed germline DNA.

The two patients with only germline DNA in their PBLs (patients no. 8 and 9), had clearly detectable rearranged Ig bands in their marrow plasma cells. Clinically, they had progressive symptomatic myeloma and were in Durie-Salmon stage IIA and IIIA. The seven patients with clonally rearranged Ig genes in PBLs were in stage IIA (two patients), IIIA (one patient), and IIIB (four patients). Thus, there was no correlation between clinical stage and DNA analysis in this small patient sample. However, one patient, (patient no. 6) did not show a new rearranged band in PBLs at the time of diagnosis when he was in stage IA, but at relapse when he was in stage IIIB, his PBLs contained clonally rearranged DNA (Fig 1). The germline J_H band is approximately 18-kb, and marrow plasma cells, at diagnosis and relapse, show a rearranged band at 13 kb. This rearranged band is only present in PBLs at relapse (Fig 1).

No patient demonstrated clonal β-TCR gene rearrangements in the PBL or plasma cell preparations.

DISCUSSION

The aim of the present study was to investigate whether significant numbers of peripheral blood lymphocytes are part of the malignant clone in multiple myeloma. The detection of clonal Ig and TCR gene rearrangements has been used to demonstrate the clonal origin of other B and T cell–derived tumors.[22-24] We have used these same gene probes to demonstrate that clonal antibody gene rearangements but not TCR gene rearrangements occur in the PBLs of most patients with multiple myeloma. These results indicate that peripheral blood B lymphocytes but not T lymphocytes are part of the malignant clone in multiple myeloma.

These data confirm the work of other investigators who demonstrated peripheral blood monoclonal B cells by either abnormal surface membrane κ/λ light-chain ratios or positive surface immunofluorescence with antiidiotypic reagents.[2-5,25,26] However, these results contradict those of Pilarski et al,[9] King and Wells,[7] and Gobbi et al[8] who failed to find monoclonal B lymphocytes in the peripheral blood of myeloma patients. Pilarski et al[9] studied only three patients in remission and used antiidiotypic reagents. In our study, we only studied patients with active disease. It is interesting to note that the patient with the lowest tumor burden at diagnosis (patient no. 6) only demonstrated involvement in the peripheral blood after the development of clinical relapse with a marked increase in tumor burden. King and Wells[7] studied two patients with active myeloma but with fairly small serum M spikes and two patients in clinical remission.

Table 1. Clinical Characteristics and Southern Blot Analysis

Patient No.	Isotype	Antibody Genes in PBLS			β-TCR Genes in PBLs and Plasma Cells
		Heavy Chain			
		Constant Region	J_H	Light Chain	
1	Gκ	R	R	R	ND
2	Gκ	ND	ND	R	G
3	Gκ	R	R	R	G
4	Gκ	ND	R	R	G
5	Gκ	ND	R	R	G
6	Gκ				
	At diagnosis	G	G	G	G
	At relapse	R	R	R	G
7	Aλ	ND	ND	R	ND
8*	Aλ	G	G	G	ND
9*	Gλ	ND	G	ND	G

Abbreviations: R, rearranged DNA identical to bone marrow plasma cells; G, germline or unrearranged DNA; ND, not done.

*Both these patients demonstrated antibody gene rearrangements in marrow plasma cells.

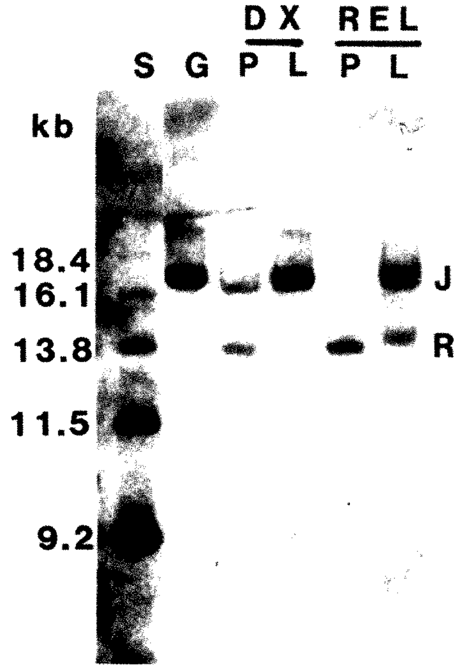

Fig 1. PBL and plasma cell DNA blot hybridization with IgJ$_H$ probe in patient no. 6. All lanes were loaded with a total of 10 μg of *Bam*HI-digested DNA from PBLs, plasma cells, or granulocytes. S, concatemerized plasmid DNA (monomeric length, 2.3 kb) used as a molecular size standard; G, granulocyte DNA, a germline control; P, bone marrow plasma cell DNA; L, PBL DNA; DX, at diagnosis; REL, at relapse; J, germline J$_H$ band; R, rearranged J$_H$ band.

These investigators used a rosette assay to detect idiotype-bearing cells after vigorous washing and failed to demonstrated PBLs bearing an endogenous idiotype when using this technique. This negative result may also reflect the low tumor burden of their patients and the insensitivity of this assay. Gobbi et al[8] used pokeweed mitogen–stimulated peripheral blood lymphocytes from multiple myeloma patients to demonstrate the polyclonality of these cells by using κ/λ ratios. However, a small but significant number of monoclonal cells may not be detected by this technique. It is also possible that the malignant PBLs may not respond to mitogenic stimulation. In contrast to the aforementioned techniques, Southern blot analysis is exceptionally sensitive. Most investigators estimate that as low as a 1% involvement of a cell mixture can be identified by this technique.[27,28]

In our study, we have not shown clonal TCR gene rearrangements in any of our patients. These results would suggest that clonal T cells do not exist in multiple myeloma as has been reported by other workers.[6] However, these investigators used antiidiotypic antisera to demonstrate T cell involvement in a single patient with myeloma. It is well known that there is marked expansion of T cells that bear Fc receptors for the idiotype,[29,30] and these "clonal" T cells were probably T cells with idiotype bound to their Fc receptors.

The common acute lymphoblastic leukemia antigen (CALLA) has been demonstrated on the surface of bone marrow cells in patients with myeloma.[31,32] Because CALLA is normally thought to be present on B lymphoid cells during early stages of differentiation, these data suggest an oncogenic event in myeloma occurring in similar early precursor cells. The presence of pre-B lymphoid cells that express homologous idiotype in two patients with IgA myeloma[33] also supports this notion. We are presently using antibody gene analysis to determine whether earlier stages in B cell differentiation have clonal expansion of cells.

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Correlation of Chromosome Abnormalities With Histologic and Immunologic Characteristics in Non-Hodgkin's Lymphoma and Adult T Cell Leukemia-Lymphoma

By the Fifth International Workshop on Chromosomes in Leukemia-Lymphoma

To correlate cytogenetic findings with histology and immunophenotype, 260 newly diagnosed patients with non-Hodgkin's lymphoma (NHL) and 31 with adult T cell leukemia/lymphoma (ATL) were studied at the Fifth International Workshop on Chromosomes in Leukemia-Lymphoma. Clonal chromosome abnormalities were found in 85% of cases of NHL and in all cases of ATL. The most significant associations of histology with cytogenetics involved the 8q24 and 14;18 translocations. Of 23 NHL patients with an 8q24 translocation, 20 (87%) had small noncleaved cell (SNC) lymphoma. Of 57 NHL patients with a t(14;18)(q32;q21), 37 (65%) had a follicular lymphoma, and at least 13 others (23%) had a diffuse lymphoma of follicular center cell origin. Five others including two who also had an 8q24 translocation had SNC lymphoma; tumors in these patients lacked the monomorphic features seen in those with only an 8q24 translocation. The most striking associations of immunology with cytogenetics involved rearrangements of 14q11-13, abnormalities of 1p, trisomy 3, and trisomy 12. Abnormalities of 14q11-13 were correlated with T cell disease. Among 138 NHL and 24 ATL cases that lacked involvement of 8q24, t(14;18), or rearrangements of 14q11-13, abnormalities of 1p and trisomy 3 were significantly associated with the T cell phenotype ($P < .01$) and trisomy 12 with the B cell phenotype ($P < .01$). No karyotype difference was found between Japanese ATL and non-Japanese T cell lymphomas except that +3 occurred more often in the former ($P = .06$). Although the 8q24 translocations in 23 patients were distributed relatively equally among geographic regions (United States, ten; Europe, ten; Japan, three), the t(14;18) in 57 patients was much less frequently seen in Japan than in the other parts of the world (United States, 31; Europe, 21; Australia, four; Japan, one). Thirty-nine chromosome bands had breaks in five or more cases; a number of protooncogenes and genes related to lymphocyte function located in these bands are likely to be involved in the development of lymphoma. Thus, besides confirming the close association of particular chromosome abnormalities with histology, we have identified a number of new associations that merit molecular analysis.

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DATA ON CHROMOSOME ABNORMALITIES in non-Hodgkin's malignant lymphoma (NHL) are accumulating rapidly, and some of them have been correlated with specific histological and immunologic phenotypes.[1-9] However the varying histologic classifications used in different institutions have posed a serious problem in correlating specific abnormalities with histology. Moreover, the complicated chromosome abnormalities often observed in lymphoma have sometimes resulted in different interpretations of the same abnormality. Finally, possible geographic or racial differences in the incidence of various chromosome abnormalities have prevented previous studies by single institutions from showing the whole spectrum of the chromosome patterns in lymphoma.

Although all preceding Workshops on Chromosomes in Leukemia had dealt with the correlation between the chromosome pattern and clinical features in leukemia,[10-13] the Fifth Workshop (see Appendix 1) considered the chromosome abnormalities present in NHL and in adult T cell leukemia-lymphoma (ATL) prevalent in southern Japan.[14] This report describes correlations between chromosome abnormalities and the histological and immunologic aspects of lymphoma. Only cases with cytogenetic and histologic studies on the same tumor tissue obtained before any antineoplastic therapy were accepted.

Analysis of the workshop data not only confirmed the importance of certain specific chromosome abnormalities such as the 8q24 translocations and the translocation between 14 and 18 but also indicated the morphological spectrum of cells with such abnormalities by using the working formulation (WF).[15] The data confirmed the significance of the abnormalities in the proximal long arm of chromosome 14 in T cell lymphomas including ATL and trisomy 12 in B cell lymphomas. Furthermore, this analysis provided further evidence for the involvement of the short arm of chromosome 1 and trisomy 3 in T cell lymphomas. A number of recurring chromosome breakpoints involved the location of known protooncogenes or lymphocyte-specific genes; others were found in bands not presently associated

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See Appendix 1 for individual participants.

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with such genes and thus identified other regions that may be important in the genesis of specific lymphomas.

MATERIALS AND METHODS

Cases of NHL with banded chromosome analysis on lymph node or other tumor tissue performed before therapy and before the end of 1983 were collected before the workshop. Karyotypes and histology slides on each case were submitted along with detailed clinical data. Cases with ATL were collected from some Japanese centers; for ATL, patients with chromosome studies only on blood or bone marrow were accepted if they were positive for anti–ATL-associated antigen (ATLA) antibody.

The submitted karyotypes were initially reviewed at the workshop headquarters in the Saitama Cancer Center (The karyotypes were reviewed at the headquarters before the workshop by R. Abe, Y. Kaneko, N. Maseki, M. Sakurai, J. Takeuchi), and again by the preworkshop karyotype review committee (R. Abe, S. Abe, G. Alimena, D.C. Arthur, R. Berger, O.M. Garson, A. Hagemeijer, D.K. Hossfeld, Y. Kaneko, and F. Mitelman), which finalized the interpretations. Karyotypes were designated according to the ISCN (International System for Human Cytogenetic Nomenclature), 1978.[16] Clonality of the abnormalities was determined by using criteria of the First Workshop.[10] All clinical, pathological, and cytogenetic data were computerized, and processed data were made available for the workshop.

The histology slides, usually accompanied by slides of cytology, were reviewed individually by five hematopathologists (C. Berard, G. Frizzera, M. Kikuchi, K. Nanba, and T. Suchi) who then met before the workshop and finalized the diagnosis according to the WF (Table 1).[15] For this workshop, diffuse lymphomas with medium-sized cells exhibiting nuclear polymorphism were also included in category E, ie, malignant lymphoma (ML), diffuse, small cleaved cell (DSC). Category J, ML, small noncleaved cell (SNC), was divided into three subcategories, ie, Burkitt, non-Burkitt polymorphic, and not otherwise specified (NOS). The results of the pathological analysis have been reported elsewhere in detail.[17] For ATL, only cases for which histology slides from tumor tissue were submitted were included within the NHL group; ATL cases for which only bone marrow and/or blood smears were available were considered separately.

Each institution studied the immunologic phenotype of lymphoma cells according to its usual procedure, using cell suspensions and/or tissue sections. Sheep erythrocyte rosette formation and surface immunoglobulin determination were generally applied. The monoclonal antibodies used varied among institutions, but the Leu or OKT series were commonly used to identify T cells and B1, B2, BA-1, and BA-2 to identify B cells.

Table 1. A Working Formulation of NHL for Clinical Usage

A. Small lymphocytic (SL)	G. Diffuse large (DL)
B. Follicular small cleaved (FSC)	H. Immunoblastic (IBL)
C. Follicular mixed (FM)	I. Lymphoblastic (LBL)
D. Follicular large (FL)	J. Small noncleaved (SNC)†
E. Diffuse small cleaved (DSC)*	K. Miscellaneous (MISC)‡
F. Diffuse mixed (DM)	

*For the workshop, diffuse lymphoma with medium-sized cells exhibiting nuclear polymorphism were included in this category.

†This category was subdivided into monomorphic (Burkitt's), polymorphic (non-Burkitt's), or NOS.

‡This category includes composite, mycosis fungoides, histiocytic, and extramedullary plasmacytoma.

Data from Non-Hodgkin's Lymphoma Pathologic Classification Project.[15]

Statistical analysis was made by the chi-square or Fisher's exact test.

RESULTS

Two hundred sixty adequately studied cases of NHL were accepted for analysis. There were 149 males and 111 females, including 202 whites, seven blacks, and 51 Orientals. The ages ranged between 1 and 86 (median, 56 years). In addition, 31 Japanese (Three ATL cases submitted from Kochi, Japan, by Y. Shiraishi are included in the study) patients (21 males and ten females; ages, 29 to 76; median, 54) with ATL whose karyotypes were analyzed from blood were also accepted. Because of the short observation period (a median follow-up of 9 months), data on survival are not included in this report.

Correlation of Karyotype With Histologic and Immunologic Features in NHL and ATL

Modal chromosome number. Two hundred twenty-two (85%) of the 260 patients with NHL and all 31 patients with ATL had clonal chromosome abnormalities. Thus, 38 (15%) of the NHL cases had a normal karyotype in cells obtained from the tumors. Modal chromosome numbers of the lymphomas by histologic category are shown in Table 2. Among 253 NHL and ATL cases with abnormalities, 92 (36%) had a modal chromosome number of 46. The second most frequent modal number was 47 (61 cases), followed by 48 (31 cases), 49 (16 cases), and 50 (nine cases). Only 13 patients had a modal number between 51 and 61, and ten had a polyploid mode. There were only 21 hypodiploid cases, of which 18 had a modal number of 45. Among the cases with abnormalities, a modal number of 46 was most frequent in SNC (68%), LBL (50%), DSC (57%), and FSC (50%) lymphomas. However, higher modal numbers (47 to 50 chromosomes) were seen in FM (60%), FL (67%), DM (59%), DL (51%), and IBL (54%) histologic groups. Tumors that showed only a normal karyotype were distributed among virtually all histological subtypes, but they were most frequent in FSC (28%), DSC (30%), DM (25%), and LBL (38%) lymphomas.

Number of abnormal clones. Table 2 also lists the number of abnormal clones observed by histologic subtype. A single abnormal clone was detected in 207 of the 253 abnormal cases (82%); thus only a single stemline was observed in these tumors.[16] Forty cases showed two clones; except for three cases, the clones were clearly related because they had common cytogenetic changes and the more abnormal clone represented further karyotypic evolution. These tumors therefore had a stemline with one sideline.[16] Of the six tumors that had more than two clones detected, five had some common cytogenetic changes, whereas the other had one clone that lacked changes related to the other clones. Thus for the whole group, there were only four tumors that showed two or more unrelated clones.

Abnormalities in 8q24. Twenty-three (8.8%) of the NHL cases and one of the ATL cases had a translocation involving band 8q24 (Table 3). There were 17 patients with t(8;14)(q24;q32), two with t(2;8)(p11;q24), and two with t(8;22)(q24;q11). In addition to the 21 patients with 8q24

Table 2. A Summary of the Modal Chromosome Number and the Numbers of Clones by the Histological Category of NHL and ATL

	Histological Group According to the International WF											No. of Cases With Lymphoma	No. of Cases With ATL
	SL	FSC	FM	FL	DSC	DM	DL	IBL	LBL	SNC	MISC		
Modal number of the simplest abnormal clone‡													
≤45	1	1				1	5	3	4	2	1	18	3
46a	8	9	9	1	4	7	4	9	5	21	2	79	13
47-50	8	8	18	4	2	16	18	20	1	7		102	15
≥51	1		1	1		2	4	3		1		13	
Polyploid			2		1	1	4	2				10	
Abnormal Total	18	18	30	6	7	27	35	37	10	31	3	222	31
46n	3	7	3		3	9	1	5	6	1		38	
Total	21	25	33	6	10	36	36	42	16	32	3	260	31
No. of abnormal clones													
1	12	12	23	4	6	26	30	31	7	26	3	180	27
2	6	6	6*	1	1		4	5†	3	5		37	3
3 or 4				1		1†	1	1				5	1

*Two patients had two unrelated clones.

†One patient had two unrelated clones.

‡Modal number, 46a; 46n, 46 chromosomes with an abnormal karyotype; 46 normal.

abnormalities involving one of the three chromosomal bands in which immunoglobulin genes are located (ie, 14q32, 2p12 and 22q11),[18] two patients had a translocation involving 8q24 and another chromosomal band. One patient had −8,+der(8)t(8;?)(q24;?), and the other had t(8;11)(q24;q13); these two cases also had a t(14;18). One ATL case had t(8;10)(q24;q22).

Five of the 17 patients with t(8;14)(q24;q32) had this translocation as the only chromosome abnormality, and two others had either only a t(2;8) or a t(8;22) translocation (Table 4); thus 14 of 21 with standard or variant 8q24 translocations had additional chromosome abnormalities (Table 5). The most common additional change was duplication of part of 1q, always including 1q21-32, which was seen

Table 3. Correlations Between Specific Chromosome Abnormalities and Various Histological Groups in the WF of NHL and ATL

Karyotype	Histological Group According to the International WF*											No. of Cases With Lymphoma	No. of Cases With ATL	Immunologic Phenotype (Lymphoma and ATL)	
	SL	FSC	FM	FL	DSC	DM	DL	IBL	LBL	SNC	MISC			B Cell	T Cell
8q24 translocations															
t(8;14)		1					1	1		14†		17		12†	0
t(2;8)										2		2			
t(8;22)										2		2			
Other										2‡		2‡	1§	2‡	1 (1)‖
t(14;18)	1	15†	19†	3	2	2	7	2		5¶	1	57¶		37†¶	2
14q11-13 abnormalities					1	1		2	2			6	6	0	10† (5)
+3	1		2#		1**	6#		2#				14‡‡	8		
+12	4	1	2§§	1#		1#	8‖‖¶¶	3		2#¶¶		22##			
Others***	12	1	10	3	4	19	20	28	8	8	2	115	16	53	43 (23)
Normal	3	7	3		3	9	1	5	6	1		38		10	7
Total	21	25	33	6	10	36	36	42	16	32	3	260†††	31	112‡‡‡	63 (29)

*For an explanation of the groups, see Table 1.

†Chromosome abnormalities were significantly correlated with histological category or immunophenotype ($P < .01$).

‡t(8;11)(q24;q13) and −8,+der(8)t(8;?)(q24;?), respectively, which are both accompanied by a t(14;18)(q32;q21)

§t(8;10)(q24;q22).

‖Numbers in parentheses indicate cases with ATL.

¶Includes two cases with atypical 8q24 translocations (see ‡).

#Includes one case with t(14;18).

**Includes one case with 14q11-13 abnormality.

‡‡Includes three cases with t(14;18) and one with 14q11-13 abnormality.

§§Includes two cases with t(14;18).

‖‖Includes one case with t(14;18) and another with +3 and t(14;18).

¶¶Includes one case with 8q24 translocation.

##Includes seven cases with t(14;18) and two with t(8;14).

***Further details of these cases are given in Tables 5-9.

†††Fifteen cases with two or more chromosome abnormalities listed in the left column were counted only once.

‡‡‡The total is less than the simple addition of figures above because two cases with atypical 8q24 translocations‡ and a t(14;18) are counted only once.

Table 4. Thirty-one Cases With a Single Chromosome Abnormality

Abnormality	Morphology*	Immunophenotype
t(8;14)(q24;q32)	J. SNC, NOS	Bl
	J. SNC, NOS	Bl
	J. SNC, Burkitt	Bk
	J. SNC, Burkitt	Bl
	J. SNC, Burkitt	ND
t(8;22)(q24;q11)	J. SNC, Burkitt	ND
t(2;8)(p11;q24)	J. SNC, NOS	ND
t(14;18)(q32;q21)	B. FSC	Bl
	B. FSC	Bk
	C. FM	other
	E. DSC	Bk
	E. DSC	NI
+3	F. DM	T
	F. DM	T
	ATL	T
+12	A. SL	Bl
	A. SL	Bk
del(6)(p23)	F. DM	T
inv ins(6)(p23q21q13)	ATL	T
del(6)(q23)	K. MISC	T
del(6)(q23q25)	A. SL	Bk
del(16)(q22)	F. DM	NI
del(11)(q14q23)	A. SL	Bl
del(14)(q24)	A. SL	Bl
inv(14)(q11q32)	I. LBL	T
+5	F. DM	T
+18	A. SL	Bk
t(8;20)(q13;q11)	A. SL	NI
t(9;21)(p24;q21)	ATL	ND
del(5)(p13)	ATL	T
−Y	H. IBL	T

Abbreviations: Bk, B cell κ light chain; B1, B cell λ light chain; NI, not interpretable; ND, not determined.

*For an explanation of histology, see Table 1.

Table 5. Additional Chromosome Abnormalities in the Karyotypes With 8q24 or 14;18 Translocations

	Cases With	
Abnormality	t(8q24) (n = 21*)	t(14;18) (n = 57)
+7 or −7,+i(7q)	1	18†
+X	0	11†
6q− or −6,+i(6p)	1	9
+12	2	7
+21	0	6
+18q−	0	6
dup(1q) or −1,+i(1q)	4	1†

Abbreviations: i(7q), isochromosome of 7q; 6q−, del(6)(q15 or 21 or 23); i(6p), isochromosome of 6p; +18q−, +der(18)t(14;18)(q32;q21); dup(1q), partial trisomy of 1q.

*Includes 17 cases with t(8;14), two cases with t(2;8), and two cases with t(8;22).

†The difference in frequency in t(8q24) compared with t(14;18) was significant, $P < .05$.

in four patients. There were two patients with +12 and one each with −X, +7, a partial deletion of either 6q or 9p, or an unidentified chromosome segment attached to band 1p36.

Of the 23 NHL cases with an 8q24 translocation, 20 (87%) had SNC lymphoma; 11 (55%) of these 20 had SNC, monomorphic (Burkitt) including one with t(8;11)(q24;q13); eight (40%) had SNC, NOS; and one patient with −8,+der(8)t(8;?)(q24;?) had SNC, polymorphic (non-Burkitt). One patient each with a t(8;14) exhibited DL, IBL, or FSC lymphoma; thus the 8q24 translocations were significantly associated with SNC histologies ($P < .01$). Seven cases with an 8q24 translocation as the sole abnormality showed an SNC morphology. Immunologic phenotyping were available for 15 cases; all except one ATL case were B cell (Table 3).

14;18 translocation. Fifty-seven (21.9%) NHL cases had a t(14;18)(q32;q21), the most common abnormality found in the workshop (Table 3). Five cases had only the translocation (Table 4), whereas 52 (91%) had additional abnormalities (Table 5). The common numerical chromosome changes were +7 (seen in 15 patients), +X (eleven patients), +12 (seven patients), +21 (six patients), and a missing sex chromosome (in eight patients). Two patients had a +7, and two others had a +12 as the only changes; one other had both a +7 and a +12 without other additional changes. Of the 28 males with a t(14;18), nine had numerical sex chromosome abnormalities (+X in five, a mosaicism of +X/+Y in one, −Y in three); of the 29 females, 12 had numerical sex chromosome abnormalities (+X in five, −X in seven). Thus, an extra X was seen with equal frequency in both sexes, whereas an extra Y was rare. The loss of an X in females was more frequent than loss of a Y in males. Among the structural changes, an extra derivative 18q− chromosome consisting of most of chromosome 18 and the terminal segment of chromosome 14 was seen in six patients; this chromosome appeared to be a duplication of the 18q− chromosome. A partial deletion of 6q was seen in six patients; a partial deletion of 4p was seen in four; and an isochromosome of 6p or 7q or a 1p+ chromosome consisting of chromosome 1 with an unknown segment attached to band 1p36 each were noted in three patients. No patient had a duplication of 1q.

In general, additional abnormalities were more common in patients with a t(14;18) than with a t(8;14). Thus the frequency of trisomy 7 or isochromosome 7q and of trisomy X was significantly higher in the former than in the latter patients ($P < .05$). The exception was the 1q duplication, which was only seen in t(8;14) patients ($P < .05$).

Cases with the 14;18 translocation were significantly more frequently ($P < .01$) FSC (26%) and follicular, mixed, small cleaved and large cell (FM) (33%) lymphomas. Except for seven diffuse lymphoma cases in which the origin of the lymphoma cells was not specified, all others with this translocation were of follicular center cell origin. Of the five cases with no additional abnormalities, two had FSC, one had FM, and the other two had DSC lymphomas. Thus, cases with a simple t(14;18) were not invariably accompanied by a follicular histologic pattern.

Most cases (37 of 39, 95%) with a t(14;18) for which immunophenotyping data were available were B cell, but two (5%) were T cell (Table 3). One patient with a T cell phenotype had an FM lymphoma, and the other had an IBL lymphoma.

Abnormalities of 14q11-13. Six (2.3%) NHL cases and six (19%) ATL cases had abnormalities involving the proximal portion of 14q, ie, 14q11-13 (Table 6). Of these 12 cases, four had an inv(14)(q11q32), and one had a t(14;14)(q11;q32); both abnormalities showed the same breakpoints. Two patients had a del(14)(q11) as well as a partial deletion of the other 14q involving either bands 14q11 to q13 or 14q13 to q24. Five other patients had balanced or unbalanced translocations involving 14q11 or 13 and other chromosomes. Only one of the 12 patients with a 14q11-13 abnormality had an inv(14) as the sole abnormality.

Eleven patients had additional chromosome abnormalities; among the numerical abnormalities, +8, +15, +21, and −X were seen in two patients each and +2, +3, +9, +11, +16, and +19 in one each. The only recurring structural change was a 1p+ chromosome with an unknown chromosome segment attached to band 1p36 in three patients.

Five of the six NHL patients with abnormalities involving 14q11-13 had T cell–specific morphologies, ie, LBL, convoluted cell (two patients), diffuse medium-sized cell lymphoma with nuclear polymorphism, DM, or IBL, polymorphous (one patient each). The sixth patient had IBL. The 14q11-13 abnormality was not seen in FSC lymphoma. All cases with a 14q11-13 abnormality for which the immunologic data were available showed a T cell phenotype.

Trisomy 3. Of the 260 cases with NHL, 14 (5.3%) had a +3 (Table 3). In two it was the only abnormality (Table 4), and in a third case one clone had only +3 whereas other clones had +3 and additional abnormalities. In addition to a +3, three also had a t(14;18), one had a 14q11-13 translocation, and the other eight had various other abnormalities. Eight cases with ATL also had a +3, in one as a sole abnormality. None of the other seven had 14q11-13 abnormalities.

The three NHL cases with a +3 as the sole abnormality had a DM lymphoma. Three of the eight cases having a +3 but none of the three common chromosome abnormalities (8q24, t(14;18), and 14q11-13 abnormalities) also had a DM lymphoma. Thus, cases with +3 primarily had DM lymphoma ($P < .05$). Three cases with +3 and a t(14;18) had FM, DM, or IBL lymphoma, and another case with a +3 and an inv(14)(q11q32) had a diffuse medium-sized cell lymphoma with nuclear polymorphism. Excluding the four cases of +3 with a t(14;18) or a 14q11-13 abnormality, 11 NHL and ATL cases had a T cell phenotype, and only two NHL cases had a B cell phenotype. Thus, +3 cases were found significantly more often with a T cell than a B cell phenotype ($P < .01$) (Table 7).

Trisomy 12. Twenty-two (8%) of the NHL cases had a +12 (Table 3). Two had a +12 as a single abnormality, and another patient had one clone consisting of only a +12 and a related clone with an additional abnormality. Twenty of these 22 (91%) had other structural and/or numerical abnormalities. Thus, seven had a t(14;18)(q32;q21), and two had a t(8;14)(q24;q32); none had a 14q11-13 abnormality.

The three cases with only a +12 showed SL morphology, and eight of the 22 cases had DL lymphoma. Thus, there was some association of +12 with the SL and DL categories of NHL. None of the cases with ATL or lymphomas histologically categorized as T cell lymphoma had a +12. Lymphoma cells of all 13 cases having +12 for which immunologic data were available showed a B cell phenotype.

Abnormalities of 1p. Twenty-eight NHL and six ATL cases had abnormalities of chromosome 1p. Of the NHL cases, one had a t(8;14), seven had a t(14;18), two had a 14q11-13 abnormality, and 18 had other abnormalities (Table 7). Two of the ATL cases had a 14q11-13 abnormality. Of the 34 NHL and ATL cases, the breakpoint occurred in 1p36 in 17, in 1p34 in four, in 1p31-32 in seven, and between 1p22 and 1p11 in six. Thus, the breaks clustered in 1p31-36. Among the 34 cases, the other involved chromosome was identified in only one case with a complex rearrangement. The most frequent change was a translocation of an unknown chromosome segment to 1p36.

Eighteen cases had a 1p abnormality without the three common abnormalities. Fourteen had a diffuse lymphoma, most commonly IBL (seven patients), but the association was not statistically significant. Where immunologic data were available, seven of the NHL cases and all ATL cases showed a T cell phenotype; only three of the NHL cases showed a B cell phenotype. Thus, a 1p abnormality significantly more frequently had a T cell rather than a B cell phenotype ($P < .01$) (Table 7).

Other abnormalities of 14q32. In NHL, 14q32 translocations involving a chromosome band other than 8q24,

Table 6. Cases With 14q11-13 Abnormalities

Center	Age	Sex	Race	14q11-13 Abnormality	Morphology*	Immunophenotype	Anti-ATLA Antibody
Rochester	54	M	W	t(1;14)(q23;q11), +der(14)t(1;14)	F. DM	T	ND
Minneapolis	10	F	W	inv(14)(q11q32)	I. LBL	T	ND
Rotterdam	18	M	W	t(11;14)(p14;q13)	I. LBL	T	ND
Saitama	33	F	O	der(?)t(14;?)(q11;?)	H. IBL	ND	ND
Nagasaki	50	F	O	+der(14)t(14;?)(q13;?)	H. IBL	T	+
	48	F	O	inv(14)(q11q32)	E. DSC	T	+
	68	F	O	del(14)(q11),del(14)(q11q13)	ATL	T	+
	56	M	O	t(14;14)(q11;q32), +del(14)(q11q13)	ATL	T	+
	62	F	O	del(14)(q11),del(14)(q13q24)	ATL	T	+
Kochi	56	F	O	inv(14)(q11q32)	ATL	T	+
Okayama	36	M	O	inv(14)(q12q32)	ATL	ND	+
	50	M	O	t(14)t(14;?)(q11;?)	ATL	T	+

Abbreviation: ATLA, ATL-associated antigen; ND, not determined.

*For explanation of the histologic groups, see Table 1.

Table 7. Correlation Between Chromosome Abnormalities and Immunologic Phenotypes of NHL and ATL in Patients Lacking the 8q24, t(14;18), or 14q11-13 Abnormalities

Location of Abnormality*	No. of Cases With Lymphoma	No. of Cases With ATL	Immunologic Phenotype (Lymphoma and ATL)	
			B cell	T Cell
1p	18	4	3	11†
1q	27	4	12	6
2p	14	2	4	4
2q	13	4	3	6
3q	20	3	10	5
+3	10	8	2	11†
6q	32	9	14	15
7q	14	4	7	5
+7	16	1	8	2
11q	22	2	13	3
12p	11	1	4	4
+12	13	0	8†	0
13q	10	3	5	3
14q32	38	5	12	12
17q	12	1	4	4
+18	17	0	8	1
+X	17	0	8	2
No. of Patients	138	24	53	43

*Only numerical changes or locations of structural abnormalities (deletions, translocations, derivative chromosomes) that were present in more than eight evaluable cases.

†Significantly correlated with the immunologic phenotype at the .01 level.

18q21, or 14q11-13 were found in 38 cases (Table 7); the translocation partner was known in only six of them, and they were all different (1q25, 2p21, 2p15, 8q22, 11p11, and 11q13). Five ATL cases had a 14q32 translocation; three had a der(14)t(14;?)(q32;?), and the other two had either a t(5;14)(q22;q32) or a t(10;14)(q21;q32).

These 38 cases were randomly distributed among all histologic categories. Of the 19 NHL cases with the 14q32 translocation and immunologic data, 12 showed a B cell and seven a T cell phenotype.

Abnormalities 6/6q. Forty-three (17%) of the 260 cases with NHL had a deletion of the long arm of chromosome 6, either a del(6q) (37 cases) or an isochromosome of 6p, i(6p), which leads to loss of the entire long arm (six cases). The deletion was thought to be terminal in 26 cases (involving two different deletions in one case), interstitial in eight, both in one, and undefined in two cases. The distinction between the loss of all of the chromosome distal to the breakpoint (terminal deletion) and loss of some intervening segment with unequivocal retention of the telomeric portion (interstitial deletion) may be unclear. Although molecular analysis of other chromosome deletions, eg, del(5q) and del(20q), has often shown them to be interstitial, in this report, deletions without a definite q terminal region were classified as terminal. Two cases had a del(6)(q23) or a del(6)(q23q25) as the single abnormality (Table 4), and 41 (95%) had other numerical and/or structural abnormalities including a t(14;18) (eight cases), an 8q24 translocation (one case), and a 14q11-13 abnormality (one case). The breakpoint was 6q11-14 in five cases, 6q15 in 11, 6q21 in 14, 6q23 in 10, 6q24 in one, and 6q25 in five. No common chromosome segments were deleted. Deletion of 6q was also seen in seven cases with ATL, four interstitial and three terminal. The breakpoint was 6q15 in four cases, 6q21 in four, 6q23 in one, 6q25 in two. Six of the seven cases had lost a part of band 6q21.

The pathological diagnoses of the lymphomas having a del(6q) or an i(6p) included all categories of the WF except DSC. Only seven of the 43 were diagnosed as follicular lymphoma. No significant correlations were noted between the breakpoint or the size of the deleted segment of 6q and the histologic or immunologic type of NHL.

Other chromosome abnormalities. Thirty-one (12%) NHL and two ATL cases were found to have abnormalities of the long arm of chromosome 11. Only one of the NHL cases had an 11q abnormality, which was a del(11)(q14q23), without other changes. Five NHL cases also had a t(14;18), and one had a t(14;18) and an 8q24 translocation involving 11q13; none of them had a 14q11-13 abnormality. The 11q abnormalities seen in NHL included balanced translocations (five), unbalanced translocations (13), terminal deletions (six), interstitial deletions (five), an undefined deletion (one), and an isochromosome of 11q (one). Three of the five reciprocal translocations had a break in 11q11-13, and the other two had 11q21 and 11q23 breakpoints; the other chromosome involved was nos. 2, 3, 8, 9, and 19. Nine of the 13 unbalanced translocations had a break in 11q23-25, and two each had a break in 11q11-13 or 11q14-22. Of 11 defined terminal and interstitial deletions, all had a break in 11q23-25, four also had a break in 11q14-22, and none had 11q11-13 breaks. Thus in the great majority of deletions and unbalanced translocations (20/26) breakpoints clustered in 11q23-25. All interstitial deletions included bands 11q21-23. The unbalanced translocations led to a terminal deletion of q25-qter in five cases, of q23-qter in three, and q11-q15 in the remainder. Thus, nine cases with an unbalanced translocation and six with a terminal deletion lost the segment distal to either 11q23 or 11q25. Two ATL cases had either an unbalanced 11q25 translocation with an unknown donor or an 11q23 terminal deletion.

There was no significant correlation between the type or extent of deletion and histology. One case with a DM lymphoma from an ATL endemic area of Japan and two cases with ATL showed a T cell phenotype; the other 14 NHL cases (excluding those with the three common abnormalities) with immunophenotyping data showed a B cell phenotype.

Three NHL and one ATL case had a translocation or a deletion involving 7q35-36, a location where the T cell β chain receptor gene has been mapped. One of the three NHL cases showed a T cell phenotype, and the other two showed B cell or non-T, non-B phenotypes. Chromosome abnormalities involving 7p15, a location where the T cell γ chain receptor gene has been assigned, were seen in two cases with NHL and one with ATL. One of the two NHL cases showed a T cell phenotype, and immunophenotyping was not done in the other. Chromosome abnormalities involving a break in 6p23 were seen in two NHL cases with T cell phenotype (DM and DL) and one ATL case; one other case with a deletion of 6p22-24 had a B cell phenotype and SNC histology.

Twenty different abnormalities were seen as the only chromosome changes in 31 of the 291 NHL and ATL cases; ten of these have been described above. The karyotypic abnormalities as well as the histology and immunophenotype of these 31 cases are listed in Table 4.

Cases with unrelated clones. Four patients had at least two apparently unrelated clones in their tumors. Two of these patients had one clone with a t(14;18)(q32;q21) plus other abnormal chromosomes (modal numbers 54 and 90), whereas the other clone had a simple abnormality, either −1,+der(1)t(1:?)(q12;?) or −13,+der(13)t(13;?)(q22;?). Both patients had an FM lymphoma, but immunophenotyping was uninterpretable. One other patient had four clones in his DM lymphoma; three of them had 47 or 48 chromosomes with a trisomy 3, but the fourth clone had an unrelated karyotype with 51 chromosomes without a +3. In this patient again, the immunophenotype was unknown. The last patient had hyperdiploid and tetraploid clones with no common chromosome changes; the tumor was IBL lymphoma, polymorphous, with a T cell phenotype.

Correlation of Histology and Immunology With Karyotype

Tables 3 and 7 show the relationship between the chromosome abnormalities and the histology and immunophenotype of the lymphomas. The largest number of NHL patients (42) had a large cell IBL type, and the smallest number (six) had an FL type.

Karyotypes in SNC lymphomas. Of the 32 SNC lymphomas, 15 were classified as NOS, 13 as monomorphic (Burkitt's), and the other four as polymorphic (non-Burkitt's) (Table 3). In the first group, seven had an 8q24 translocation, three had a t(14;18), one had both an 8q24 translocation and a t(14;18), three had other abnormalities, and one had a normal karyotype. In the Burkitt's group, 11 had an 8q24 translocation, two had other abnormalities, but none had a t(14;18). In the third group, one had a t(14;18), and three had other abnormalities, but none had an 8q24 translocation. While all five patients with a t(14;18) (including two with both a t(14;18) and an 8q24 translocation) were more than 40 years old, those with an 8q24 translocation but no t(14;18) were generally young, with a median of 11 years.

Karyotypes in follicular (FSC, FM, and FL) lymphomas. When all 64 patients with follicular lymphoma (FSC, FM, or FL) were considered, ten had a normal karyotype, and 37 (58%) had a t(14;18) (Table 3). Among the 17 patients with chromosome abnormalities but without a t(14;18), one had a t(8;14)(q24;q32), and seven had −14,+der(14)t(14;?)(q32;?); thus, the latter tumors contained a 14q+ chromosome with an unidentified donor chromosome. Nine cases had various other chromosome abnormalities.

Immunophenotypic data. One hundred fifteen cases had the immunologic phenotype of the lymphoma cells determined only on tumor cells in suspension, eight only on tumor tissue sections, 84 on both, and four on unknown types of material. One hundred twelve cases were shown to be a B cell, 34 to be T cell, and eight to be non-T, non-B phenotype; the data were uninterpretable in 57 cases. In ATL, 29 patients had immunophenotyping on malignant cells in the blood, and all were proved to have a T cell phenotype. Thus, 146 cases with lymphoma and 29 cases with ATL exhibited either B or T cell phenotypes; the association of the immunologic phenotype and specific chromosome changes have been described in detail in the preceding sections and are summarized in Tables 3 and 7.

Lymphomas in patients under 20 years of age. Thirty-three (13%) of the 260 NHL patients were under 20 years of age. Of these, 15 had SNC lymphomas, 13 had LBL, three had IBL, and two had DL. Twelve of the 15 SNC patients had an 8q24 translocation, two had a 14q+ chromosome with an unknown chromosome segment at band 14q32, and one other had a normal karyotype. Nine of the 13 LBL patients had chromosome abnormalities, two with a 14q11-13 and three with 6q-abnormality; these abnormalities were also seen in T cell lymphomas of various histologies in adults. Four of the five patients with a 14q11-13 or 6q-abnormality showed a T cell phenotype.

Geographic Differences in Karyotypic Features

8q24 v 14;18 translocations. The geographic distribution of the karyotypic pattern in NHL was examined; attention was focused on the two most frequent specific translocations (Table 8). Thus, of the 23 patients with an 8q24 translocation, ten were American, ten European (both all white), and three Japanese; the proportion of all patients in each geographic area was 10%, 10%, and 6%, respectively. Of the 57 patients with a t(14;18), 31 were American (one black), 21 were European, four were Australian, and only one was Japanese; the proportion of all patients in each geographic area was 32%, 21%, 31%, and only 2%, respectively. Thus, the 8;14 translocation showed an even racial or geographic distribution, but t(14;18) was more frequent in the United States and Europe (or whites) than in Japan (or Japanese) ($P < .01$).

Japanese ATL v non-Japanese T cell lymphomas. The chromosome pattern of neoplastic cells from 38 patients with T cell lymphoma/leukemia in the ATL endemic area of Japan was compared with that from 21 patients with T cell lymphoma outside Japan, excluding the two patients with a t(14;18) and T cell phenotype. The Japanese patients included 31 ATL and seven others whose lymph nodes had a T cell phenotype. As shown in Table 9, 14q11-13 abnormali-

Table 8. Correlation of Chromosome Abnormalities in NHL With Geographic Region

	Cases With NHL				
Chromosome Abnormality	USA	Europe	Australia	Japan	Total
8q24 translocations	10*	10*		3	23
t(14;18)	31	21	4	1	57
14q11-13 abnormalities	2	1		3	6
Others	42	53	6	38	139
Normal	12	16	3	6	37
Total	96†	100†	13	51	260†

*One case had an atypical 8q24 translocation and a t(14;18).

†The total is less than the simple addition of the figures above because two cases with an 8q24 translocation also had a t(14;18).

Table 9. Chromosome Abnormalities in Japanese ATL and Non-Japanese T Cell Lymphomas

Abnormality	Japanese ATL* (n = 38†)	Non-Japanese T Cell Lymphomas (n = 21‡)
14q11-13	8	3
6q15-21	9	6
14q32	7	3
6p21-23	4	0
1p32-36	6	4
10q22-26	5	0
9p11-24	5	1
+3	9	1
5q11-13	6	0

*The cases were submitted from Nagasaki, Kochi, and Okayama. All 38 cases were submitted as having ATL from these centers, of which seven cases whose histology slides were also submitted were included in the 260 NHL cases and the others not accompanied by such slides were accepted as one of the 31 ATL cases.

†Includes one case with a normal diploid tumor.

‡Includes four cases with a normal diploid tumor and excludes two cases with a t(14;18) and T cell phenotype.

ties were seen in eight Japanese patients and in three non-Japanese patients. Three of the former patients and one of the latter had an inv(14)(q11q32), and two of the former had a del(14)(q11) and/or a del(14)(q11q13). Deletions of 6q having breakpoints in 6q15 or 6q21 were seen in nine Japanese and in six non-Japanese patients. Abnormalities of 14q32 and lp were seen equally in both groups of patients. The most striking difference was the frequency of +3 and of 5q11-13 abnormalities. A +3 was seen in nine Japanese and in only one non-Japanese patient ($P = .06$), and 5q11-13 abnormalities were found in six Japanese and in none of the non-Japanese patients ($P = .06$).

Recurrent breakpoints. Thirty-nine chromosome bands were the sites of five or more independent breaks; the number of breaks is listed in Table 10. Some of these breakpoints were known locations of cellular oncogenes and of genes important in lymphocyte functions.[18-24] The breakpoints clustered to regions such as 1p32-36, 6q13-25, and 11q13-25, each of which contains several protooncogenes; other breakpoints such as 14q32 and 14q11 are the sites of lymphocyte-related genes.

DISCUSSION

In this paper, we report results obtained at the Fifth International Workshop on Chromosomes in Leukemia-Lymphoma and focus specifically on the correlation between the chromosome abnormalities and the histologic diagnosis or the immunologic phenotype of the lymphomas. Although the study was retrospective and certain limitations existed, especially in the assessment of the incidence of various chromosome abnormalities, the centralized exhaustive review of the karyotypes and the consensus pathologic diagnosis using slides submitted from the same tumor were unique to this workshop. The cytogenetic and pathologic data obtained from this very large number of lymphoma cases appear to show a clear correlation between these two parameters, especially with regard to 8q24 translocations, t(14;18), and rearrangements of 14q11-13. In addition, cases with ATL submitted from a number of Japanese centers permit the comparison of the karyotypic features between this well-known endemic T cell disease and the T cell lymphomas seen in other parts of the world.

Table 10. Breakpoints Observed in Patients With NHL and ATL and Location of Various Protooncogenes or Lymphocyte Function Genes

Breakpoint*	No. of Patients	Gene†	Breakpoint*	No. of Patients	Gene†
1p36	15	FGR/SRC2	8q24	24	MYC
1p34	5	LCK	9p22	5	IFF, IFB1
1p32	5	LMYC	9q34	5	ABL
1q21	13	SKI	10q22	5	
1q32	8	TRK	10q24	5	TDT
1q42	6		11q13-14	6	BCL1, INT2
2p25	6	NMYC	11q23	13	ETS1, THY1
2p11-12	5	IGK, TGFA	11q25	8	
2q37	5		12p12-13	9	KRAS2
3p25	7	RAF1	12q24	6	IFG
3p21	10		13q34	5	
3q21	5		14q11-13	14	TCRA
3q29	10	p97	14q32	101	IGH, AKT1
5q13	7		17p11	5	
6q13	6		17q25	9	
6q15	17		18q21	62	YES1, BCL2
6q21	20	SYN	19p13	7	MEL
6q22	12	ROS	19q13	11	
6q23	8	MYB	22q11	6	IGL, BCR
6q25	9	MET			
7q22					

*Breakpoints observed in five or more cases are listed.

†IGK, Immunoglobulin κ chain gene; TCRα, T cell receptor α chain gene; IGH, immunoglobulin heavy-chain gene; IGL, immunoglobulin λ chain gene. Data are from various sources.[18-24]

Modal Chromosome Numbers

The distribution of the modal chromosome numbers were similar to those reported earlier in lymphoid malignancies including acute lymphoblastic leukemia and lymphoma.[3-5,12] When the histologic category was considered, hyperdiploid modal chromosome numbers were seen more frequently in cases with FM, FL, and DL lymphomas. These findings were related to the fact that the cells with a t(14;18) had a propensity to acquire additional abnormalities. Rather fewer cases with SNC or LBL had a hyperdiploid modal chromosome number, which seemed to reflect the rare occurrence of additional abnormalities in the t(8;14) and LBL.

Correlation of the Karyotype With Histological and Immunologic Features in Lymphoma

The workshop has confirmed the correlation of histology in B cell lymphomas with the karyotype, namely, 8q24 and t(14;18) abnormalities. Except for the two cases with atypical 8q24 translocations and t(14;18), none had more than one of the frequent abnormalities. The 8q24 translocation particularly showed a good correlation with morphology, ie, with SNC, a majority of the patients being subclassified in Burkitt's type. The cases with an 8q24 translocation but with a histology other than the typical SNC had a histology closely related to SNC. The t(14;18) was primarily associated with follicular lymphomas (FSC or FM). Many of those with a t(14;18) but with a nonfollicular pattern showed a histology of follicular center cell origin.

However, the t(14;18) was seen in five patients with SNC lymphoma, including two with both t(14;18) and 8q24 translocations. These cases were classified in the NOS or the polymorphic (non-Burkitt's) subcategory. In contrast to the young age of patients with an 8q24 translocation, these five patients were all older. Thus, these findings suggest that there are two biologically different types of SNC lymphoma and that the one with a t(14;18) arises from a germinal center, whereas lymphomas with a t(8;14) arise from outside the germinal center.

The t(8;14)(q24;q32), when present as the sole abnormality, was always associated with SNC morphology, often being diagnosed as Burkitt's lymphoma. On the other hand, cases with a simple t(14;18)(q32;q21) did not always show follicular morphology. Thus, although three of the five such cases were follicular, the remaining two patients showed a DSC morphology.

Structural abnormalities involving 14q11-13, a location where the T cell receptor α chain gene has been assigned, were associated with various morphologies of T cell lymphoma, ie diffuse medium-sized cell lymphoma with nuclear polymorphism, DM, IBL polymorphous, and LBL, and indicated the importance of the proximal 14q abnormality in the occurrence of both thymic and peripheral T cell lymphoma.

Among numerical abnormalities, +3 and +12 correlated with distinct subtypes of lymphoma, both histologically and immunologically. Thus, +3 was almost always associated with a T cell phenotype and DM lymphoma or ATL. The fact that three cases having a +12 as the single abnormality has SL morphology suggests that SL lymphoma with a +12 is another specific cytogenetic subtype of NHL. Trisomy 12 also correlated with the DL category, all of which showed a B cell phenotype. The biologic importance of +3 and +12 should be the subject of future investigation. Although these trisomies may be directly related to the expression of the immunologic phenotype, they may also be superimposed on some primary unidentified genetic changes. A significant correlation of 1p abnormalities with T cell lymphomas was also established, which suggested that genes related to T cell expression may be located on 1p. Cases with a 6p23 breakpoint and 11q abnormalities seemed to be correlated with T cell and B cell lymphomas, respectively. The significance of these associations is currently unknown.

Uneven Geographical Distribution of Some Lymphomas

Although the 8q24 translocation–associated lymphomas showed an equal racial or geographic distribution, the t(14;18)-associated lymphomas were more common in the United States, Europe, and Australia than in Japan. The uneven distribution of the t(14;18)-associated lymphoma may be related to the fact that follicular lymphoma, which is known to be common in the United States and Europe, is rather rare in Japan.[14]

Although the number of patients examined was small, there seemed to be no difference in the chromosome pattern between ATL from the Japanese endemic area and T cell lymphomas elsewhere. The only exception was an extra chromosome 3, which was seen more frequently in Japan. The only non-Japanese workshop case with a clone with a +3 as a sole abnormality had a DM lymphoma of peripheral T cell origin. This difference may be related to the fact that half of the T cell lymphomas outside Japan were LBL that were primarily composed of malignant lymphoid cells in the thymic stage of differentiation, whereas ATL is believed to be a malignant change of the peripheral T cell. Further support for this interpretation is provided by studies of human T-lymphotropic virus I–positive patients from the Caribbean; the most frequent change observed in four of six patients was a +3.[25]

Recurring Chromosome Breakpoints

One of the most exciting areas in cancer research has developed from the genetic analysis of recurring chromosome rearrangements, especially translocations. Analysis of the t(8;14) in Burkitt's lymphoma has revealed that this translocation results in the juxtaposition of a protooncogene, *c-myc*, at 8q24 and the immunoglobulin heavy (IgH) chain gene at 14q32 (Table 10).[26,27]

Study of the t(14;18) has shown that IgH is involved with a different gene of unknown function called *bcl-2*.[28] More recently, analysis of a t(8;14)(q24;q11) in T cell CLL has revealed a rearrangement analogous to that seen in Burkitt's lymphoma; in T cells, the gene for the α chain of the T cell receptor is moved next to *c-myc*.[29,30] In this workshop report we have presented further evidence for the frequent involvement of certain chromosome regions in recurrent rearrangements, namely, 1p32-p36, 6q13-q25, and 11q13-q25. A number of protooncogenes are located within these same chromosome segments. Based on the aforementioned research, the search for involvement of some of these protooncogenes in the appropriate rearrangements is likely to be productive.

This workshop has provided further evidence that patients with lymphoma can be classified according to the specific chromosome abnormalities present in the tumors and that these patients present group-specific features. Further efforts to define additional cytogenetic groups will help to clarify the histologic characteristics and immunologic phenotypes of the various subtypes of lymphoma. The fact that cases with a normal karyotype were distributed among most histologic groups and about equally between immunologic phenotypes may indicate that in most if not all such cases we were studying the karyotype of the normal rather than the lymphoma cells. Prospective studies will help to find additional recurring abnormalities, to determine further the distribution of various abnormalities among races or geographic regions, and to evaluate the prognostic significance of the karyotype in NHL and ATL.

ACKNOWLEDGMENT

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APPENDIX 1

The Fifth International Workshop on Chromosomes in Leukemia-Lymphoma was held in Ranzan, Saitama, Japan, October 11 to 15, 1984. The Workshop was organized by M. Sakurai, Y, Kaneko, and N. Kamada. The participants were S. Abe, Sapporo, Japan; G. Alimena, Rome; D.C. Arthur, Minneapolis; C.W. Berard, Memphis; R. Berger, Paris; C.D. Bloomfield, Minneapolis; G. Borgström, Helsinki; A. de la Chapelle, Helsinki; G. Dewald, Rochester, MN; G. Frizzera, Minneapolis; O.M. Garson, Melbourne; H.M. Golomb, Chicago; A. Hagemeijer, Rotterdam, The Netherlands; D.K. Hossfeld, Hamburg, Germany; N. Kamada, Hiroshima, Japan; Y. Kaneko, Ina, Saitama, Japan; M. Kikuchi, Fukuoka, Japan; U. Kristoffersson, Lund, Sweden; S.D. Lawler, London; C. Mecucci, Leuven, Belgium; F. Mitelman, Lund, Sweden; K. Miyamoto, Okayama, Japan; K. Nanba, Hiroshima, Japan; P. Philip, Copenhagen; R. Pierre, Rochester, MN; J.D. Rowley, Chicago; N. Sadamori, Nagasaki, Japan; M. Sakurai, Ina, Saitama, Japan; A.A. Sandberg, Buffalo; T. Suchi, Nagoya, Japan; L. Teerenhovi, Helsinki; H. Van Den Berghe, Leuven, Belgium; and H. Yamamoto, Hiroshima, Japan. N. Maseki, K. Shimamura, R. Abe, J. Takeuchi, M. Ohta, and H. Nagatsuma served as the workshop headquarters staff.

The workshop participants acknowledge the important contributions of their respective collaborating pathologists: M.E.F. Prins, Rotterdam, The Netherlands; J. Rodhain, Brussels-Leuven, Belgium; A. Louwagie, and C. Peeters, Leuven, Belgium; L. Ruco, Rome; K. Franssila, Helsinki; P.M. Banks, Rochester, MN; D. Variakojis, Chicago; and G. Flandrin, Paris; B. Gusterson, London; M. Åkerman, Lund, Sweden.

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Precommitted Erythroid Cells Enriched in Cultures of Suboptimally Induced Friend Erythroleukemia Cells

By Enrico DiMambro, Maria Galanti, and Stuart B. Levy

In the presence of suboptimal inducing amounts of dimethylsulfoxide or hexamethylenebisacetamide, a major proportion of uncommitted murine erythroleukemia (MEL) cells was found to be precommitted or primed for commitment, which was demonstrated by their rapid commitment to terminal differentiation when recultured for short periods of time (three to six hours) with fresh inducer. These same cells did not commit if left in the original inducer-containing media or if replated in fresh media without inducer. The two inducers could be interchanged in the priming and postpriming period without affecting the commitment event. However, hemin, an agent that induces hemoglobin synthesis without commitment, showed no ability to enhance commitment of a primed cell population. The rapid commitment of primed cells was inhibited by 12-0-tetradecanoylphorbol-13-acetate and cordycepin but not by cycloheximide. The latter finding indicated that this rapid inducer-dependent commitment event required new RNA synthesis but not new protein synthesis. The precommitment state was lost within six hours of the growth of cells in the absence of inducer but could be sustained longer if cells were incubated in cycloheximide. These studies characterize a precommitment state not previously described and one that appears during chemically induced differentiation in the absence of metabolic inhibitors. The stabilization of these precommitted cells by cycloheximide suggests that the reversibility of precommitment involves new protein synthesis. These findings show that MEL cells proceed to terminal differentiation by accumulating unstable products that must be maintained by the inducer until the final commitment event.

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CHEMICAL INDUCTION of erythroid differentiation of murine erythroleukemia (MEL) cells in vitro has facilitated characterization of the commitment process.[1] At a certain point in the differentiation event, MEL cells no longer require the continued presence of the inducer and are termed committed. Analysis of the commitment process, including the use of various inhibitors, has led to the discovery of multiple factors and steps involved in the commitment decision.[2-4]

Recently cell fusion experiments have distinguished between two kinds of inducer-mediated cellular events, one that is MEL cell specific and the other not cell type specific, that act synergistically to produce commitment.[4-6] A protein factor that is not cell type specific and is thought to arise from a disturbance of DNA metabolism has been identified[6] and shown to be active in the differentiation process. MEL cell–specific factors, on the other hand, are less well defined but seem to arise from inducer-mediated transmembrane signals that can be negatively modulated by tumor promoters and have their life span prolonged by cycloheximide treatments.[5] Paradoxically though, the conversion of these signals into factors that are active in reprogramming the developmental fate of MEL cells requires protein synthesis.[5,7,8]

We have been interested in the events that occur during the period preceding terminal commitment.[9] In this communication, we show that suboptimal amounts of inducer will produce cells that although yet uncommitted, are primed for rapid commitment in response to further contact with the same or even a different inducer. This enhanced commitment occurs within three hours of recontact with the inducer, is inhibited by cordycepin and 12-0-decanoylphorbol-13-acetate (TPA) but not by cycloheximide, and is lost within six hours in the absence of inducer. These primed cells represent cells at a reversible intermediate step(s) in the commitment process that have not previously been described.

MATERIALS AND METHODS

Cell cultures. Friend MEL cell lines PC-4, a subclone of the parent line 745 (provided by D. Housman, Massachusetts Institute of Technology), and C7D-Cl, subcloned from cell line C7D,[10] were maintained in culture as described.[9]

Induction studies. Induction was performed on freshly seeded logarithmically growing cells at a density of 10^5 cells/mL in the presence of designated amounts of inducers ie, hexamethylenebisacetamide (HMBA), dimethylsulfoxide (DMSO), or hemin (all obtained from Sigma Chemical Co, St Louis). Cell viability was assessed by trypan blue exclusion. Cell death never exceeded 5% for the first four days of culture. For the induction enhancement studies, cells were grown in inducer at different concentrations for 24 to 72 hours (depending on the experiment) and then either diluted into fresh inducer-containing medium or collected by centrifugation, washed in fresh medium, and resuspended in medium with or without inducer. The number of hemoglobin-producing cells was assayed by the benzidine test[11] 48 hours after replating in fresh medium.

Commitment assays. Commitment to erythroid differentiation was assayed in 1.8% methylcellulose (Fisher Co, Medford, MA) prepared according to Ogawa and Leary[12] and used as outlined by Murate et al.[7] The colonies were examined 72 to 120 hours after plating. The plating efficiency (total colonies greater than single cell/total number of colonies × 100) of uninduced cell lines was always greater than 85%. Colonies were scored for both size (ie, number of cells in each colony) and percentage of cells within each colony that was benzidine-positive (B+). The degree of commitment

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was defined, on a percentage basis, as the ratio of B+ colonies (<64 cells in size) to the total number of colonies scored.

In the augmentation experiments, cells previously grown for 48 to 72 hours in an inducer were introduced into similar or different concentrations of fresh inducer. The cells were either collected by centrifugation (500 g for six minutes) and then resuspended into new medium or diluted directly into the new medium; the results were identical. After incubation for various periods of time (three to 24 hours) in fresh inducer, the cells were collected by centrifugation (500 g for six minutes) and resuspended in 0.6 mL of fresh medium from which 0.3 mL was plated into methylcellulose. By this procedure, recovery and cell viability were >95%.

In the inhibition studies, cordycepin (Sigma; final concentration, 20 µg/mL) prepared according to Murate et al,[7] TPA (10 ng/mL; Sigma), and cycloheximide (0.5 µg/mL; Sigma) were added to the cultures at different times in relation to the inducers. In experiments looking at clonal expansion of a subset of cells, the assays were performed in methylcellulose supplemented with different amounts of inducer.

RESULTS

Augmented inducibility of MEL cell lines. Two different MEL cell lines were compared for their response (percent B+ cells) to two inducers, DMSO and HMBA. Both cell lines exhibited similar inducibility with HMBA, but C7D-Cl was more responsive to DMSO (Fig 1).

The induction response was tested when the cells were treated with suboptimal amounts of DMSO (1%) and then replated after 24, 48, or 72 hours into fresh medium and the same amount of inducer. Although replating caused an increased induction at all the times tested, the effect was most dramatic when done at 48 or 72 hours (Fig 2). PC-4 cells left untouched in 1% DMSO contained 15% B+ cells, whereas cultures transferred at 48 or 72 hours into fresh inducer showed 50% to 55% B+ cells when tested at 120 hours.

The same effect was noted when cells were grown in suboptimal levels of HMBA (2 mmol/L) and then replated into fresh medium containing fresh inducer at the same concentration (Table 1). In general the replating produced a greater than twofold increase in B+ cells. This enhanced induction was most marked when low concentrations of inducer were used during the initial contact (Fig 3). When the concentration of HMBA went above 2.5 mmol/L and that of DMSO above 1.5%, the enhancement by replating was dramatically less due to increased induction of the entire cell population. Cells that had not previously grown in inducer remained unaffected by replating into fresh inducer (<1% to 2% B+). When cells grown for 48 hours in inducer-containing medium were collected by centrifugation and resuspended back into the original medium, they showed the same induction level as that of untouched inducer-treated cells.

In these experiments, the cell concentration was reduced three- to fourfold by dilution and replating into fresh medium. In other studies in which the cell concentration was unchanged by the replating procedure, the same increase in

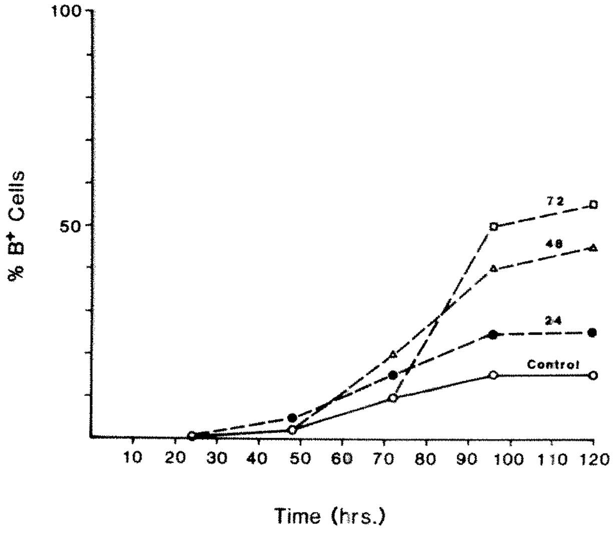

Fig 2. Effect on induction of replating cells suboptimally treated with inducer. PC-4 Cells were incubated in media containing 1% DMSO. One plate (control, solid lines) was kept untouched for the entire test period. At 24, 48, or 72 hours the cells were removed, diluted 1:4 into fresh media with inducer, and placed in separate plates (dashed lines). The percent B+ cells was determined during the entire 120-hour period.

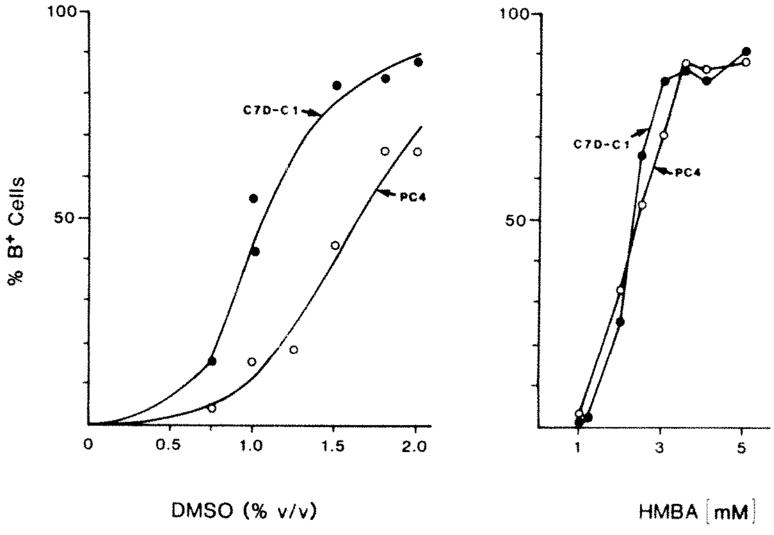

Fig 1. Induction kinetics of C7D-C1 and PC-4 MEL cells in liquid culture with DMSO or HMBA. The percentage of cells that stained with benzidine (% B+) was assayed after 96 hours of treatment. The points represent an average from two to eight separate experiments.

Table 1. Augmentation of Induction of MEL Cell Lines

	Initial Inducer Concentration	Postplating Inducer Concentration	Induction Level of Unreplated Cultures (% B+)	Induction Level of Replated Cultures (% B+)
C7D-C1	1% DMSO	1% DMSO	40.0	60.0
	2 mmol/L HMBA	2 mmol/L HMBA	25.6	63.1
PC-4	1% DMSO	1% DMSO	15.4	37.0
	2 mmol/L HMBA	2 mmol/L HMBA	32.9	71.6

Cells were incubated for 48 hours with suboptimal amounts of inducers and then were replated into fresh media containing the same inducer concentrations. The percent B+ cells was evaluated at 96 hours, 48 hours after replating. These results are representative of many independent experiments.

induction was evident (data not shown). These findings indicated that the augmented induction was not related to a change in cell density.

Augmented induction in replated cultures results from recruitment of inducer primed cells. To determine the basis for the augmented induction upon replating, we replated cells primed for 48 hours with 1% DMSO into 1.8% methylcellulose with or without inducer. At 6, 24, 36, and 48 hours, the plates were scored for growth and benzidine positivity. The augmentation in induction experienced by the primed cells in inducer-treated liquid cultures was faithfully reproduced in inducer-containing methylcellulose (Fig 4A). Furthermore, because there was an increase of B+ colonies (Fig 4B), the replating procedure caused a significant recruitment of primed cells for differentiation along the erythroid pathway. This recruitment does not take place in the absence of inducer (control, Fig 4) or with unprimed cells (data not shown).

In these experiments, the dual scoring of colony for both size and benzidine phenotype allowed a clonal comparison of growth rates. Over the initial 24- to 36-hour period, DMSO-treated cells did not show any difference in growth kinetics from that of control cells in methylcellulose containing or not containing inducer. Later, however, it became apparent that regardless of the growth rates of the cultures as a whole, a substantial portion of the B+ colonies grew slower than the benzidine-negative ones. These findings provide further evidence that the rapid augmentation in induction in replated cultures is not due to a clonal expansion of already induced cells but is due to a fresh inducer-specific mediated event(s) on primed cells.

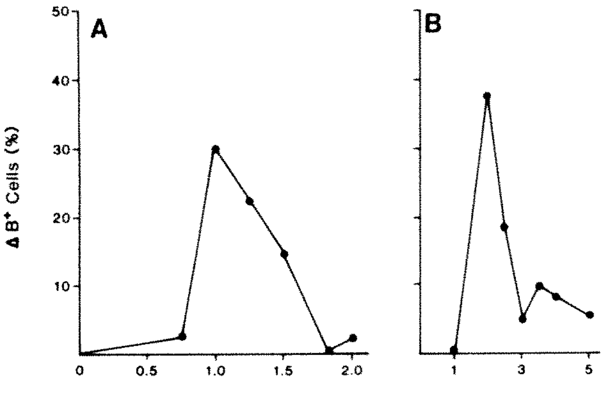

Fig 3. Effect of inducer concentration on augmentation of induction. MEL cells were grown for 48 hours at varying concentrations of inducer and then replated in fresh medium and inducer at concentrations identical to that in the initial growth period. The percent B+ cells was assessed 48 hours after replating. The difference (Δ) in percent B+ cells present in the replated culture as compared with those left in the initial inducer medium is plotted against the inducer concentration.

HMBA and DMSO, but not hemin, can be interchanged without loss of augmentation of induction. To establish whether the augmented induction was inducer specific or could be effected by ones other than those used initially (ie, in the priming period), we replated 48-hour cultures primed either with 1% DMSO or 2 mmol/L HMBA in fresh medium containing 100 µmol/L hemin or varying concentrations of DMSO or HMBA. Exchanging DMSO or HMBA in the postpriming period gave the same augmented induction relative to using the same inducer in the priming and postpriming periods. No additional augmentation was found by increasing the concentration of the inducer in the postpriming period (Table 2). No augmentation was found when primed cells were incubated with hemin (100 µmol/L) (Table 2). These findings showed that the primed state was responsive to more than a single inducer but did appear to distinguish between those inducers that lead to commitment (DMSO, HMBA) and hemin, which does not.[13]

Augmented induction in replated cultures reflects an augmentation in commitment. To determine whether the augmented induction of B+ cells was related to terminal differentiation, we examined the production of B+ colonies in methylcellulose from 48-hour DMSO-primed cultures after replating for various times (12 to 24 hours) in fresh medium and 1% DMSO. After growth for 96 hours in methylcellulose, colonies that displayed benzidine positivity and contained less than 64 cells in size were considered terminally committed.[7] We noted that replating DMSO-primed cultures at 48 hours always led to an increase in number of committed cells (Table 3). In the absence of inducer (ie, when primed cells were replated in fresh medium alone) there was little change in commitment relative to cultures that were not replated.

To define the minimum time needed for the augmentation, we examined the effect of short periods of contact with fresh inducer after the 48-hour priming period. Replated cultures required only three to six hours of additional contact with inducer to achieve augmentation in commitment (Fig 5). This augmentation was maximal by 12 hours with no further change with 24 hours of additional contact with suboptimal amounts of inducer (Fig 5, Table 3).

The lower level of commitment in cultures not replated

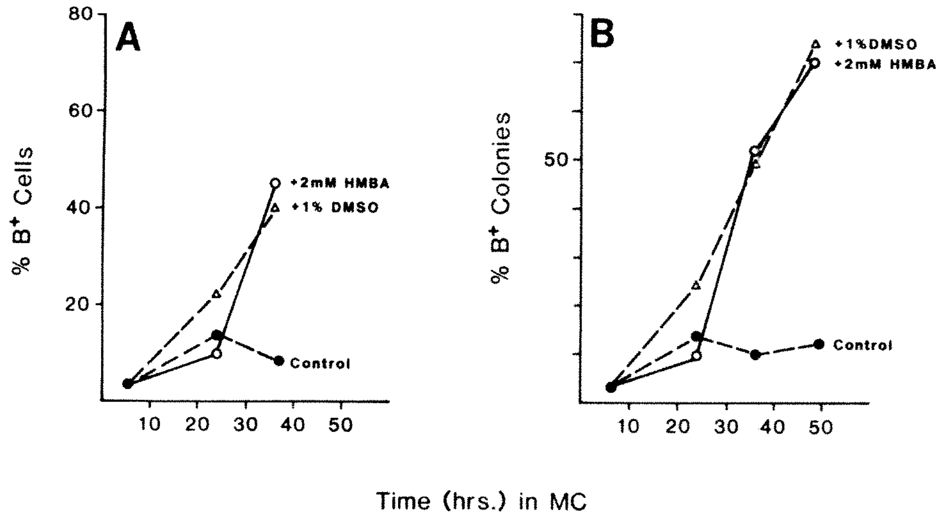

Fig 4. Augmentation in induction of primed cultures upon replating into inducer-containing methylcellulose. One percent DMSO-treated PC-4 cells from 48-hour-old cultures were imbedded in 1.8% methylcellulose at approximately 10^6 cells/mL. The methylcellulose contained either no inducer (control), DMSO (1%), or HMBA (2 mmol/L). The plates were benzidine stained at 6, 24, 36, and 48 hours of growth in methylcellulose. At these times the plates were scored for both the percent B+ cells (A) among the total cells in at least 300 colonies and the percent B+ colonies (B).

could relate to depletion of factors present in the medium needed for terminal differentiation. This possibility was tested by substituting the fresh inducer in a fresh medium combination with (*a*) fresh inducer in medium aged at 37°C for 60 hours (*b*) fresh inducer in 0.2-μm–filtered spent medium taken after 60 hours from untreated cell cultures, or (*c*) medium and DMSO incubated for 60 hours without cells and without the addition of fresh inducer. None of these substituted media altered the level of augmented commitment (data not shown). The findings suggest that it is the inducer not the media that needs to be fresh to drive primed cells into a committed state. They also suggest that DMSO is altered or depleted in media containing cells but remains unchanged if incubated alone in medium.

The level of enhanced commitment is related to the concentration of inducers in priming but not the postpriming period. The increase in newly committed cells was greater when MEL cells were primed with low (suboptimal) amounts of inducer when presumably a greater proportion of precommitted cells were present (Fig 6). At 1% DMSO an additional 20% B+ colonies were seen in PC-4–plated cells.

Table 2. Effect of Exchange of Inducers in Postpriming Period on Induction of PC-4 Cells

Priming Condition	Postpriming Condition	Induction Level of PC-4 Cells (% B+ Cells)
2 mmol/L HMBA	Fresh medium	29.3
	Untouched	32.9
	2 mmol/L HMBA	54.5
	1% DMSO	57.3
	1.25% DMSO	56.0
	1.5% DMSO	60.0
	100 μmol/L hemin	27.0
1% DMSO	Fresh medium	11.5
	Untouched	15.4
	1%	37.0
	1.25%	40.0
	2 mmol/L HMBA	35.0
	5 mmol/L HMBA	25.0
	100 μmol/L hemin	18.0

Priming and postpriming periods were 48 hours each. Results presented are representative of several independent experiments.

This number of newly committed cells remained present despite an increased total commitment of the population up to 1.8% DMSO. At 2% DMSO the replating did little to augment commitment when the culture was over 80% committed. Increasing the postpriming concentration of inducer over that used during priming did not lead to further augmentation (data not shown).

Proliferative potential of primed cells. We compared the colony size distribution of cells from primed cultures resuspended for six hours in fresh medium without or with fresh inducer. The newly committed cells that resulted from the fresh inducer contact gave rise to B+ colonies that were composed of two to 64 cells. As compared with control cells, cells incubated with fresh inducer for this short period did not show a change in benzidine-negative colonies in this size range. These findings indicate that the newly committed cells do not arise from a more efficient inducer-driven hemoglobinization of already terminally committed cells.

TPA and cordycepin but not cycloheximide, if present in the postpriming period, will inhibit the augmented commitment. To gain an insight into the biochemical processes that force replated primed cells into a state of commitment, we incubated 1% DMSO-primed cultures for six hours in fresh medium containing either 1% DMSO or 2 mmol/L HMBA and either 10 ng/mL TPA, 20 μg/mL cordycepin, or 0.5 μg/mL cycloheximide before plating in methylcellulose. Irrespective of the inducer used in the postpriming period, TPA and cordycepin completely eliminated the augmentation of commitment (Fig 7). In contrast, cycloheximide had

Table 3. Augmentation of Commitment of PC-4 Cells Primed for 48 Hours With 1% DMSO

Additional Time in Original Medium or in Fresh Inducer (h)	Percent Commitment*		
	Original Medium	Fresh Medium + 1% DMSO	Difference
0	26	—	—
12	24	50.5	26
18	21	51	30
24	21.3	44.9	23

*Percentage of B+ colonies assayed after 96 hours in methylcellulose. The results are representative of several independent experiments.

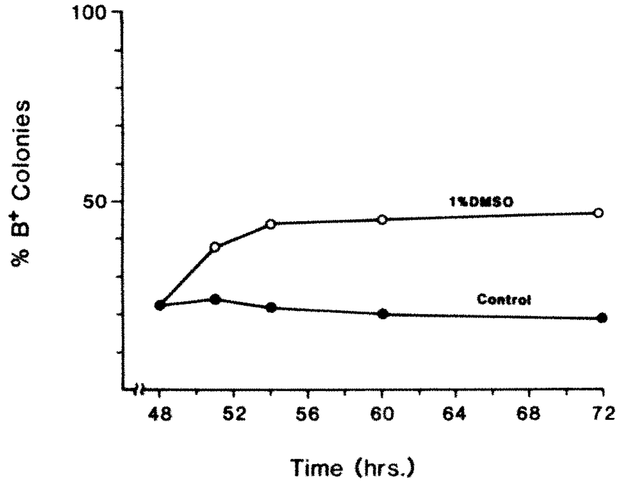

Fig 5. Kinetics of augmented commitment upon replating. PC-4 cells primed for 48 hours with 1% DMSO were resuspended in fresh media with 1% DMSO for 3, 6, 12, or 24 hours before embedding in methylcellulose. The percent B+ colonies were assayed after 96 hours.

no effect on the augmentation; at this concentration, cycloheximide completely inhibited cell growth (data not shown). These latter findings suggest that during the postpriming period the rapid inducer-mediated augmented commitment required RNA but not new protein synthesis. Furthermore, the findings indicate that either the synthesis of the RNA or its eventual translation or degradation is modulated by a TPA-sensitive step(s).

Stability of primed state; effect of cycloheximide. We examined the stability of the primed state of PC-4 cells treated for 48 hours with 2 mmol/L HMBA. If the cells remained in the priming medium, as shown previously (Fig 2), they retained the ability to become rapidly committed with fresh inducer during the 12-hour test period (Fig 8A). Upon transfer to fresh medium without inducer, the primed cells lost their ability to respond to fresh inducer within 12 hours (Fig 8B). This rather rapid loss (ie, half-life of approximately six hours) was partially prevented by the addition of cycloheximide (0.5 μg/mL) to the fresh medium (Fig 8C).

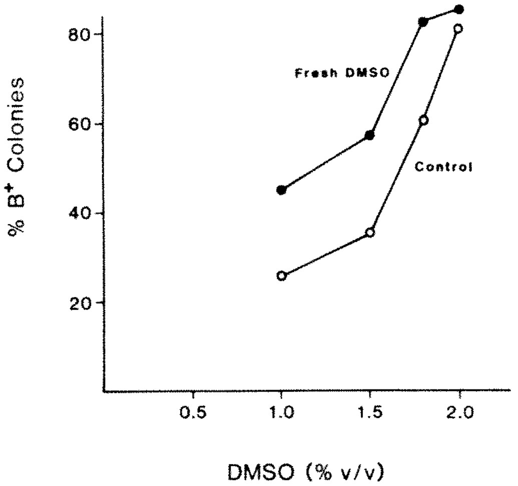

Fig 6. Effect of initial inducer concentration on number of primed cells. PC-4 cells were primed for 72 hours with varying amounts of DMSO and then replated in fresh medium with the same amount of fresh inducer for 12 hours or left untouched for an additional 12 hours (control). The percent B+ colonies in the cultures was assessed after 96 hours of growth in methylcellulose.

DISCUSSION

Intrinsic in the hypothesis that inducer-mediated commitment is a multistep process[2,3] is the idea that there are in inducer-treated MEL cell cultures cells that have experienced several biochemical events of the differentiation process, are not fully committed, but have the ability to become so if appropriately stimulated. These cells, which can be defined as precommitted or primed for commitment, may exist in several states depending on the number of events in the commitment process that they have completed. Examples of MEL cells that are progressively closer to terminal commitment are those that retain a memory of a prior exposure to inducer[14] and those that will commit rapidly in the presence of inducer after the removal of inhibitors of commitment: cordycepin,[15] amiloride,[16] or dexamethasone.[7] There are also those cells that no longer require inducer to complete commitment but can be kept from doing so by TPA.[17] Primed cells have also been described in cultures of human myeloid leukemia cells that after contact with one inducer, retinoic acid, responded to the differentiation effects of another.[18]

We have enriched MEL cell cultures for precommitted cells by their growth in suboptimal amounts of inducers. This method does not require treatment of cells with inhibitors of the commitment process, cell fusions, or extensive subcloning for particularly responsive cell populations.[7] However, these cells, like MEL cells released from the block by various inhibitors, also require brief additional fresh inducer contact to complete the commitment process. The ease with which these cells are obtained make them a useful tool in the analysis of the rapid inducer-driven molecular events that operate in the later stages of the commitment process. We found that these precommitted cells both differed from and resembled commitment-inhibited cells and MEL cell fusions. They are different from dexamethasone-treated cells in that their augmented commitment was inhibited by cordycepin but not cycloheximide[7] (Fig 7). These cells are thus at a step in the terminal commitment process that does not require the immediate synthesis of new proteins but, presumably, the synthesis of a relatively stable poly-A–containing RNA molecule. In this way they resemble cells blocked in commitment by cordycepin,[15] although it is unclear whether they are biochemically identical; comparable inhibitor studies were not performed on cordycepin-treated cultures. Moreover, our studies showed that the commitment-related RNA remains stable or is continuously made while untranslated during six hours of cycloheximide treatment or as long as inducer is present. Our experiments also indicate that a TPA-sensitive step may either precede the inducer-driven RNA synthesis or be involved in metabolizing the newly induced RNA(s) or in producing the target(s) to which the product of this RNA(s)

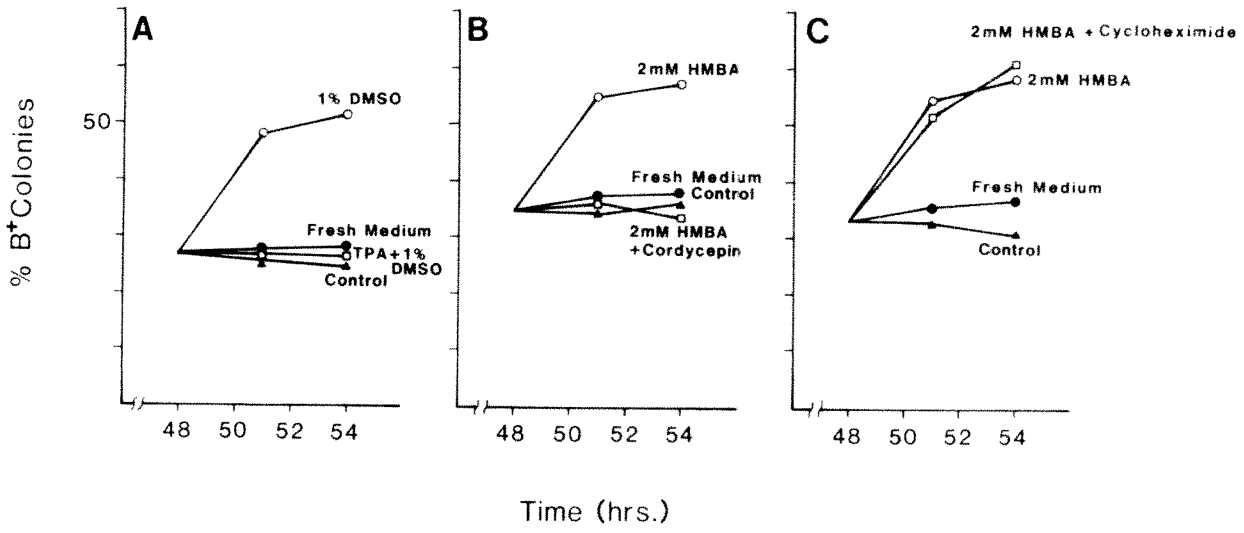

Fig 7. Effect of TPA, cordycepin, or cycloheximide on augmentation of commitment. PC-4 cells were primed for 48 hours in 1.0% DMSO (A) or in 2 mmol/L HMBA (B, C). The cells were then either left untouched (control) or collected and incubated in fresh medium or medium with fresh inducer with or without different inhibitors. (A) Fresh medium with fresh 1% DMSO ± 10 ng/mL TPA. (B) Fresh medium with fresh HMBA with or without cordycepin (20 μg/mL). (C) Fresh medium with fresh HMBA ± 0.5 μg/mL cycloheximide. At three and six hours after incubation, the cells were collected and imbedded in methylcellulose (1.8%) at a concentration of 10^6 cells/mL. The level of commitment (ie, percent B+ colonies) was evaluated after 120 hours of growth in methylcellulose.

is directed. This effect of TPA is distinct from what inhibits final commitment[17] because the removal of TPA did not cause primed cells to become committed. The primed cells, therefore, also provide a potential system for studying the events that precede this inducer-independent (TPA-sensitive) commitment step. On the basis of its rapidity of onset, this inducer-dependent event is akin to the rapid inducer-mediated TPA-inhibitory step demonstrated in the MEL cell fusion partner.[6]

Primed cells also resemble the MEL cell fusion partner[5] as well as dexamethasone-blocked cells[7] in being unstable once inducer contact is terminated (Fig 8). However, we showed that the primed state could be stabilized by inhibiting protein synthesis with cycloheximide, which is reminiscent of the

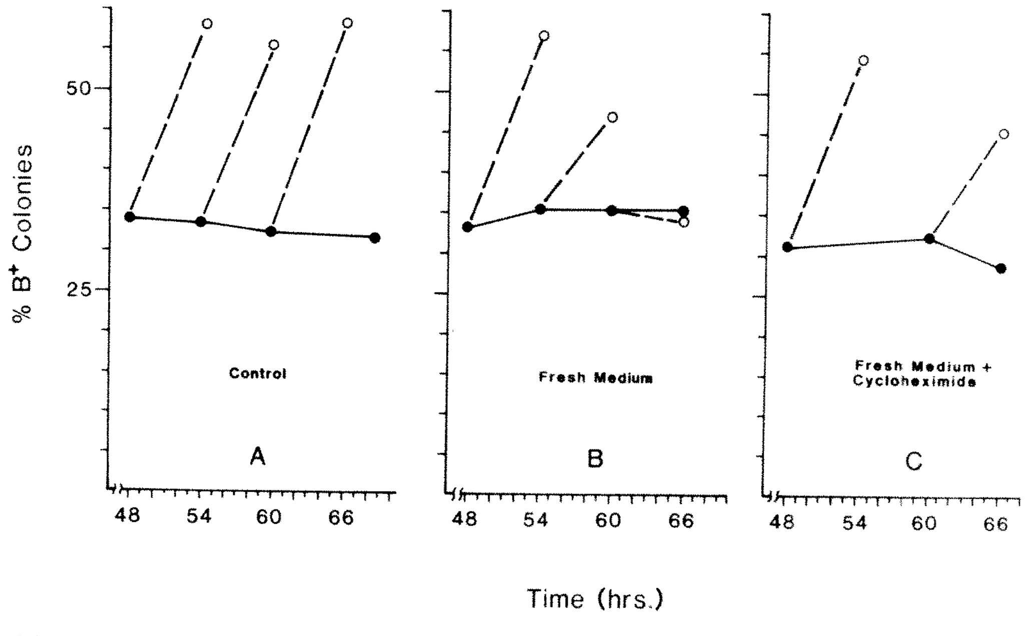

Fig 8. Stability of primed cells and effect of cycloheximide. PC-4 cells primed for 48 hours with 2 mmol/L HMBA were collected by centrifugation and immediately resuspended either in (A) their own medium, (B) fresh medium, or (C) fresh medium with cycloheximide (0.5 μg/mL). At 0, 6, and 12 hours after replating, a portion of the cells were recentrifuged and rechallenged with fresh medium containing 2 mmol/L HMBA for six additional hours. At the end of this period, the cells were imbedded in methylcellulose and the percent B+ colonies recorded after 110 hours. Key ●—●—, level of commitment in control cultures not replated in fresh inducer; O--O, level of commitment in cultures that were incubated in fresh medium and 2 mmol/L HMBA.

effect of cycloheximide on the postulated inducer-dependent transmembrane activity in cell fusion experiments.[6] This finding implicates a protein product in reversing the precommitment state.

The evaluation of the commitment assays in terms of both colony size and benzidine phenotype allowed us to better define several characteristics of the primed cells. First, these cells do not seem to derive exclusively from cells that in the primed cultures give rise only to sectored colonies (ie, mixed benzidine phenotype colonies). The proportion of mixed colonies remained low (ie, <5% of the total colonies) whether the primed cultures were left undisturbed or briefly recontacted with fresh medium with or without inducer or metabolic inhibitors. Second, primed cells are not all at the same physiological state; commitment takes place in cells that can undergo from one to six cell divisions. Third, it is unlikely that brief inducer recontact only leads to increased hemoglobinization of already committed cells because in the undisturbed, replated, or metabolic-inhibited primed cultures we never found a significant portion of cells that underwent a limited proliferation without expressing the B+ phenotype. Moreover, hemin, which induces hemoglobin synthesis,[13] did not increase commitment (Table 2).

The dependence of primed cells on fresh inducer, and not fresh medium to complete the commitment, suggests that the inducer (DMSO or HMBA) is altered or depleted after 24 to 48 hours in the presence of MEL cells. Previous studies with radiolabeled DMSO showed accumulation of the label in MEL cells.[19] With higher (optimal inducing) concentrations of inducer, enough active inducer must still be present throughout the three-day period needed to complete the inducer-mediated events. The fact that spent inducer maintains the primed state (Fig 8A) suggests that even if some of the inducer is altered or depleted sufficient amounts are present to maintain cells in a primed state.

Although the events leading to commitment appear to be sequential, these data together with those presented by others[3,6,7,14,15] suggest that the different components of the terminal commitment event do not need to be synchronously made to be effective. Nor do their activities appear to be limited to a particular step in the commitment process, but instead they seem required and must be inducer maintained throughout a precommitment period. Commitment, therefore, emerges from an accumulation of these factors, independently synthesized but interdependent, all of which remain inducer driven and required until the final commitment event.

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Mechanism of Erythrocyte Aggregation and Sedimentation

By Thomas L. Fabry

Unstirred suspensions of erythrocytes form stable spherical aggregates of uniform size. The radius of the spheres depends upon the suspending medium and the hematocrit. Erythrocyte suspensions will undergo sedimentation only after these aggregates are formed. Aggregation is a two-step process: first, erythrocytes associate in long chains (rouleau formation). Next, these chains form spheres of uniform size. The requirements for this well-defined process are an electrolyte and a neutral or negatively charged macromolecule in the solution and a metabolically active red cell. If these conditions are not met, red cells either will not aggregate at all or will form amorphous aggregates. Rouleau formation and sedimentation are inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, an inhibitor of anion transport, but not by ouabain, a cation transport inhibitor. The kinetics of erythrocyte sedimentation reflects the aforementioned mechanism: no sedimentation occurs during rouleau formation. Once the spheres of uniform size are formed, they will settle according to the Einstein-Stokes equation. In this model, parameters of sedimentation kinetics are the delay before sedimentation starts, the rate of sedimentation in the steady state, and the radius of the sedimenting aggregate. The radius can be calculated from the rate of fall of the aggregates and agrees well with the microscopically observed radius. It is inversely proportional to the hematocrit, which explains the elevated sedimentation rates in anemia.

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THE ERYTHROCYTE SEDIMENTATION RATE (ESR) is a useful qualitative empirical index of nonspecific disease activity.[1] Its clinical usefulness is, however, limited by three major problems: (*a*) quantitative interpretation is not possible, (*b*) there is a poor correlation with measurements of other acute phase reactants, and (*c*) it is paradoxically low in several diseases (eg, certain forms of inflammatory bowel disease,[2] sickle cell disease,[3] and possibly in congestive heart failure[4]). Increased ESR has been attributed to increased amounts of some plasma proteins (fibrinogen,[5] etc), but again there is only very poor quantitative correlation. Expressing the sedimentation rate as the amount of sedimentation at one hour is only meaningful if the process occurs uniformly over time. Any deviation from zero-order kinetics means that more than one parameter must be used to describe the process.

Chien[6] laid the foundation for interpreting the rheology of red cell suspensions. He established the need for electrolytes and macromolecules to be present in the suspending medium for rouleau formation to occur. Perelson and Wiegel,[7] using techniques of statistical mechanics, showed that the average rouleau size increases as the adhesion energy between erythrocytes increases. Samsel and Perelson[8] developed an elegant kinetic model for rouleau formation and pointed out that as rouleaux grow large they form rings and branching aggregates. Evans and Buxbaum[9] and Buxbaum et al[10] quantified the surface affinities of erythrocyte membranes for macromolecules and related them to aggregation.

Quantitative interpretation of the sedimentation rate is possible by developing a kinetic model based on the underlying mechanism. This may make erythrocyte sedimentation a useful tool for investigating the pathophysiology of some diseases. Furthermore, quantitative interpretation of sedimentation may provide an inexpensive screening test of plasma protein and erythrocyte membrane abnormalities in some less common disorders.

MATERIALS AND METHODS

Blood specimens anticoagulated with K_3EDTA were obtained from healthy volunteers and were processed immediately after collection. Red cells were suspended in autologous plasma to obtain different final hematocrit values (Hct). For the inhibition experiments, washed red cells were incubated with 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (0.1 mg/mL) for 30 minutes at 37°C. This is the same concentration of DIDS that is used to inhibit the chloride/bicarbonate antiporter of the erythrocyte membrane. After incubation the cells were washed with isotonic saline containing 10 mmol/L glucose and then reconstituted with plasma.

Ouabain (Sigma Chemical Co, St Louis) was dissolved in water (0.01 mol/L stock solution; final concentration of incubation mixtures, 0.1 mmol/L). DIDS was prepared in HEPES buffer immediately before use. Other chemicals were obtained from standard sources.

Sedimentation experiments were performed in Kimble disposable 200-mm Westergren tubes at pH 7.4 and at 25 ± 2°C. Experiments were completed within three hours of sample preparation to avoid artifacts from irreversible aggregation of red cells. To obtain the photomicrographs, erythrocyte suspensions were allowed to sediment in rectangular cross-section tubes 0.2 × 5 or 0.3 × 5 mm and 100 mm long (Vitro Dynamics, Rockaway, NJ). The kinetics of sedimentation in this tube is the same as in a Westergren tube filled to 100 mm. The tubes were placed flat side down on the microscope stage in different phases of the sedimentation, and photographs were taken at 4× and 10× magnification.

Curve fitting and statistical calculations were carried out by using the computer programs SAS[11] and Statgraphics.[12] The Einstein-Stokes equation was used as derived by Hiemenz.[13]

RESULTS

Kinetics of erythrocyte sedimentation and the effect of Hct. Erythrocyte sedimentation follows an S-shaped curve with time (Fig 1 and Table 1). There is a delay in the start of sedimentation; during this time the rate of sedimentation is

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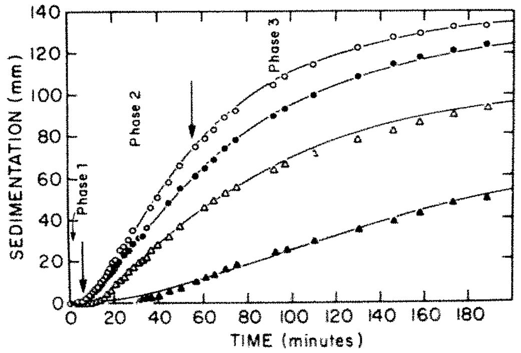

Fig 1. Sedimentation as a function of time at different Hct values. ○, Hct of 26; ●, Hct of 28; △, Hct of 34; ▲, Hct of 45.

zero (phase 1 of the curve). The next segment (phase 2) is linear: sedimentation continues at a constant rate. Finally, in phase 3 the rate levels off to zero: sedimentation is completed.

At different red cell concentrations (Hct range, 10 to 50) the shape of the curve is qualitatively the same; however, there is a shift to the right as the Hct increases (Fig 1): the delay (phase 1) increases and the slope in phase 2 decreases. This results in slower sedimentation at a higher Hct value.

Effect of membrane transport inhibitors on sedimentation. DIDS, an irreversible inhibitor of anion transport, inhibits both rouleau formation and sedimentation in a dose-dependent manner. Ouabain (0.1 mmol/L) has no effect on the sedimentation rate. Table 2 shows the slope of the sedimentation curve and the ESR as a function of inhibitor concentration.

Direct observation of aggregation during sedimentation. In phase 1 the homogeneous red cell suspension forms a three-dimensional network (Fig 2) that, late in this phase, rearranges to form spheres of equal size. This is a sudden change. It can be observed with the naked eye in the Westergren sedimentation tube: the homogeneous cell suspension suddenly becomes granular in appearance, with the spheres visible at the sides of the tube. In phase 2 these spheres sediment at a steady rate (Fig 3). Figure 4 is a photomicrograph of the interface between the sedimented cells and the plasma. The sedimenting entities are the spheres formed from the rouleaux. The extent of aggregation does not change during phase 2. To demonstrate that the red cells form fairly stable aggregates during the steady phase of sedimentation (ie, after the delay period) the ESR tube was gently inverted (ie, turned upside down). Sedimentation continued at the same steady rate without a second delay period.

Table 2. Effect of Anion and Cation Transport Inhibitors on Sedimentation

Inhibitor	Concentration (μmol/L)	Slope	ESR
Blank		0.6	28
DIDS	1	0.5	23
DIDS	10	0.1	5
Oubain	100	0.6	23

DISCUSSION

The delay in red cell suspension sedimentation suggests either aggregation (ie, red cells will not settle until aggregates of sufficient size are formed) or cooperatively (ie, the rate constant for the sedimentation increases with time). Red cell sedimentation kinetics is not compatible with either. Simple aggregation would require the sedimentation rate to increase with an increasing concentration of erythrocytes (Hct); in fact, the opposite occurs. As shown in the Appendix, the equation describing sedimentation kinetics does not fulfill the criteria for cooperativity.

Aggregation of red cells to form uniform spheres. The mechanism of rouleau formation has been extensively investigated.[14] Our data allow further elaboration of the mechanism of aggregation.

Red cells repel each other because of their negative surface charge from sialic acid residues. At the same time they are attracted by the van der Waals forces, which are electrodynamic in nature. The balance of these two forces determines the most stable arrangement of red cells in an electrolyte solution in the absence of other forces. Assuming a discoid shape for red cells, van Oss and Absolom[15] calculated that at an intercellular distance of 103 nm there is a minimum of -4.6×10^{-12} ergs/cell in the potential energy distribution of erythrocytes suspended in isotonic saline. The distance of the closest approach for erythrocytes increases with decreasing electrolyte concentration because the repulsive force (ζ potential) increases with decreasing ionic strength and the van der Waals forces are unchanged. (This explains why there is no rouleau formation in the absence of electrolytes, eg, in sucrose solutions.) Therefore, in the presence of electrolytes, the most stable configuration of erythrocyte suspensions is a chain of cells attached by their flat surfaces. However, this energy is small relative to the energy from Brownian motion (kT); therefore, cells would align only transiently but would not form stable structures. For this reason red cells will not form rouleaux if the suspending solution contains electrolytes only.

For rouleau formation to occur, macromolecules must be present in the suspending medium.[16] Chien and Jan[17] showed

Table 1. Measured and Derived Parameters Describing the Curves in Fig 1

Hct	a_0	a_1	a_2	Slope (100 × mm/s)	Delay (min)
25.8	152 ± 2	2,645 ± 56	11 ± 2	2.4	6.5
28.6	152 ± 2	3,081 ± 82	29 ± 3	2.1	8.0
35.8	123 ± 4	4,629 ± 239	31 ± 9	1.6	13.0
44.7	80 ± 10	26,324 ± 1,545	−38 ± 35	0.5	30.0

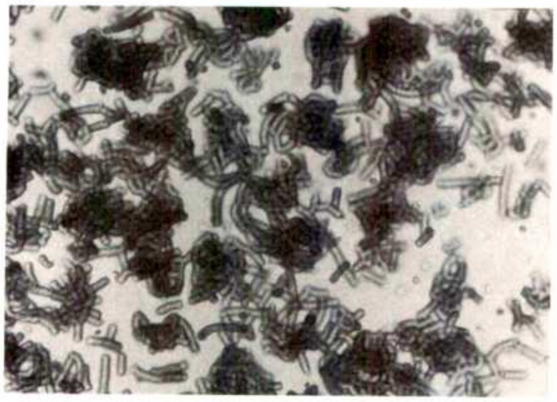

Fig 2. Photomicrograph (original magnification ×40; current magnification ×16) of sedimenting erythrocytes in a 0.2-mm-diameter tube. The rouleaux are starting to form spheres: phase 1 of Fig 1.

that increasing the length of a macromolecule (dextran) will increase the intercellular spacing of the red cells in the rouleau. He concluded that the macromolecule actually bridges two erythrocytes and only elongated macromolecules of sufficient length can serve this function. For example, fibrinogen but not albumin meets the shape requirements to cross-link erythrocytes. Therefore, sedimentation will not take place in serum. It has not been made clear until now, however, that the macromolecule has to be either neutral or negatively charged (as opposed to positively charged).

The receptor (binding) site for the macromolecule on the erythrocyte membrane has not been identified. We have shown that DIDS, an inhibitor of anion transport, interferes with formation of the aggregates in either plasma or dextran. DIDS at similar concentrations inhibits the chloride-bicarbonate exchange across the red cell membrane.[18] This suggests that the macromolecule binds at the anion transport site. DIDS also reacts with lysine residues on the membrane surface. If the fibrinogen binding involves these lysine residues, then DIDS would interfere at this point. Against this latter possibility is the fact that there are 10^6 anion transport sites (band 3 protein)[19] per erythrocyte and about 10^4 fibrinogen molecules are bound to each cell at equilibrium.[20] Because fibrinogen binding is weak, the 100:1 ratio of binding sites to bound molecules is the right order of magnitude. In the case of rouleau formation, the two ends of the macromolecule bind to adjoining erythrocytes to form the aggregates.

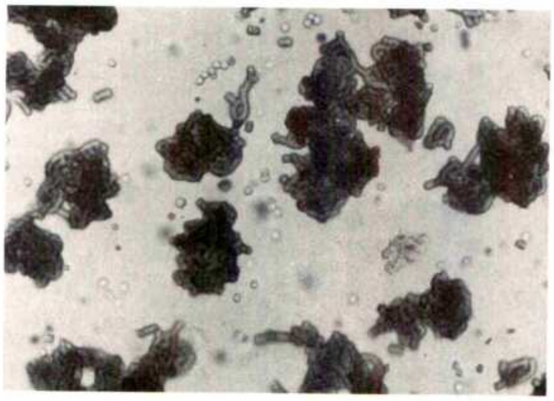

Fig 3. Photomicrograph (original magnification ×40; current magnification ×16) of sedimenting erythrocytes in a 0.2-mm-diameter tube: phase 2 of Fig 1. Note the uniform size of the spheres.

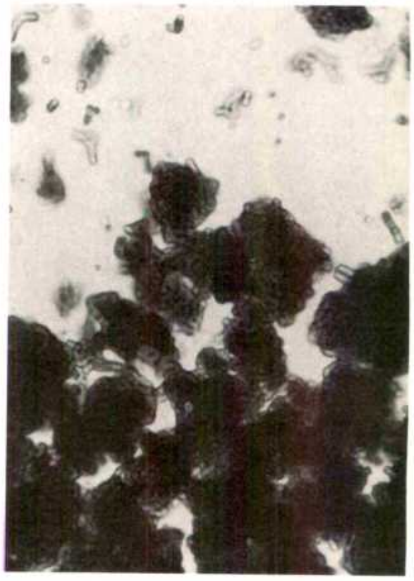

Fig 4. Photomicrograph (original magnification ×40; current magnification ×16) of sedimenting erythrocytes in a 0.2-mm-diameter tube at the interface between the clear plasma and the sedimented red cells: phase 3 of Fig 1. Note that the sedimenting entities are spheres formed from rouleaux and not individual red cells.

In contrast to the aforementioned mechanism, positively charged macromolecules are not able to bridge the erythrocytes in this fashion; they react with the sialic acid residues and form amorphous aggregates.[21] This process is not inhibited by DIDS.

After the formation of the rouleaux, the chains form spheres of uniform size (Fig 3). The driving force may be the reduction of the surface free energy. Uniform size is probably the result of competition between the attractive forces between cells and the hydrodynamic forces that tend to reduce the size of the spheres.

The spheres contain not only erythrocytes but also the macromolecules binding them together. This is in agreement with the findings of Janzen et al[22] who demonstrated binding of fibrinogen to red cell surfaces. It also explains why increased Hct values do not increase the rate of sedimentation: the formation of the spherical aggregates is limited by the availability of fibrinogen or other plasma macromolecules linking the erythrocytes.

In summary, the mechanism of red cell aggregation to spheres of uniform size proceeds via the following steps: (*a*) electrostatic forces draw erythrocytes together to about 100 nm, with the discoid shapes lining up parallel to one another. This requires the presence of electrolytes. (*b*) If a neutral or negatively charged macromolecule of length greater than

100 nm is present, it will bind to the anion transport sites of adjoining erythrocytes. The energy of this binding will stabilize the parallel configuration of red cells, and rouleaux are formed. (*c*) Rouleaux rearrange to form spheres, which reduces the surface free energy. (*d*) The kinetics of sedimentation must be described by at least two independent parameters (eg, delay before sedimentation starts and steady-state rate of sedimentation). For a mathematical analysis see the Appendix.

Clinical correlation: correction for Hct. In clinical practice there are two reasons for variations in the sedimentation rate: (*a*) its dependence on Hct and (*b*) its dependence on the plasma protein concentration. The latter makes it useful as an empirical index of disease activity. In Fig 5 the radius of the sedimenting sphere (which is directly proportional to the true rate of sedimentation) is shown as a function of Hct and plasma protein concentration. The radius is increased by either decreasing the Hct or increasing the plasma protein concentration. Therefore, a change in Hct masks the effect of changing plasma protein concentration and composition. Because the steady-state sedimentation (slope in phase 2) in the range of Hct 10 to 50 is a linear function of Hct (Fig 6), correction can be made by interpolating to a set value, eg, to an Hct of 40. This eliminates the variations in the sedimentation rate due to the changes in Hct. In practice a good estimation of the steady-state sedimentation can be obtained by measuring the amount of sedimentation at 30 and 60 minutes and multiplying their difference by 2 (this way the sedimentation will have a value that is similar to the ESR). Because the value of the slope approximates 0 when Hct = 55, one can obtain the value at an Hct of 40 from the value measured at any other Hct by the relationship: Sed (Hct = 40) = Sed (observed) × (55 − 40)/(55 − Hct), (equation 1), where Sed (observed) is the sedimentation observed at the given Hct and Sed (Hct = 40) is its value corrected to Hct 40.

Table 3 shows both Sed (observed) and ESR and their values corrected to Hct 40 by using equation 1. In a larger series of patients with inflammatory bowel disease we obtained identical results. Clearly Sed (Hct = 40) provides an index of disease activity that is fairly independent of Hct.

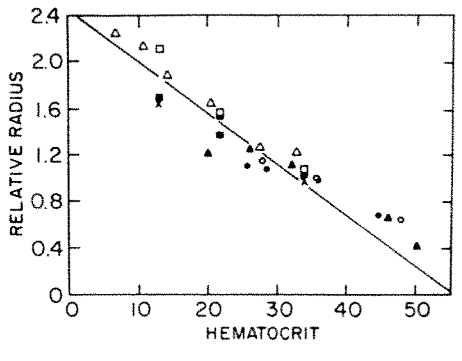

Fig 5. Radius of the spherical aggregates at different Hct values. Erythrocytes are suspended in autologous plasma and serum mixtures. The third coordinate is the fraction of plasma in the suspending medium.

Table 3. Hct Correction of Sedimentation by Using Equation 1

Hct	Sed (observed)	ESR	Sed (Hct = 40)	ESR (Hct = 40)
25.8	80	80	41	41
28.6	70	65	40	37
35.8	46	42	38	33
44.7	22	11	36	18

Sed (observed) and ESR are experimentally determined (see the text). Sed (hct = 40) and ESR (Hct = 40) are the calculated corrected values.

APPENDIX

By using SAS for modeling, the best functional form of the kinetics of erythrocyte sedimentation is as follows: sedimentation = $a_0 \times t^2/(a_1 + a_2 \times t + t^2)$ (equation 2), where t is the sedimentation time and the a's are experimental parameters. For a cooperative phenomenon $a_2 = 0$.

Two additional descriptive parameters can be derived from the three parameters in equation 1. The first is the delay before sedimentation starts, ie, the length of phase 1. The second is the slope of the linear part of phase 2. These two entities are related to the parameters in equation 2: the slope is the value of the first derivative of equation 2 when the second derivative is equal to zero and the delay is the x-intercept of the straight line originating from the inflection point of this curve and having the calculated slope. Alternatively, these can be directly measured from the plot of the curves in Fig 1. Table 1 gives the values for these parameters for the curves from Fig 1.

The linear part (phase 2) of the sedimentation results as the spheres formed from the rouleaux fall in a viscous medium. The radius R of this sphere can be calculated from the Einstein-Stokes equation: $R^2 = 9\, v\, \eta/2(\sigma_2 - \sigma_1)g$ (equation 3), where η is the plasma viscosity, σ_1 is the plasma density, and σ_2 is the red cell density. The densities and viscosities are literature[23] values, and v is the measured rate of sedimentation in the linear phase (phase 2).

These radii can also be measured in the photomicrographs. Figure 6 shows both the calculated and measured values for the radii as a function of Hct for seven samples for a series of different Hct values. Because the concentration of the bridging macromolecules was different in each series, the radius is expressed relative to that at Hct 35. There is an excellent agreement between calculated and measured values.

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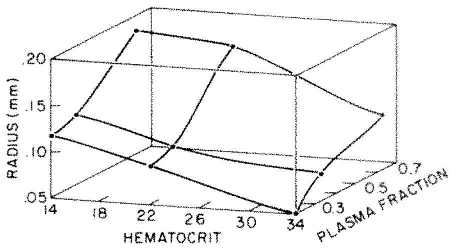

Fig 6. Radius of spherical aggregates as a function of Hct. The seven different symbols represent different blood samples. The radius is expressed relative to that observed at Hct 35.

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Localization of a Collagen-Interactive Domain of Human von Willebrand Factor Between Amino Acid Residues Gly 911 and Glu 1,365

By Michel Kalafatis, Yukihiro Takahashi, Jean-Pierre Girma, and Dominique Meyer

A collagen-binding domain of von Willebrand factor (vWF) has been identified in the central part of the molecule by comparing the binding properties of vWF and *Staphylococcus aureus* V-8 protease–generated vWF fragments with collagen. The binding of purified human vWF to human type III collagen was found to be specific. At saturation, 38 to 50.2 μg of vWF bound per milligram of collagen. Scatchard plots derived from binding isotherms demonstrated the presence of at least two classes of binding sites. Purified vWF was digested with *S aureus* V-8 protease into two complementary fragments (SpIII and SpII). SpII, the C-terminal end of vWF (amino acid residues 1,366 to 2,050), was totally devoid of affinity for collagen. Contrarily, purified SpIII, the N-terminal part of vWF (residues 1 to 1,365), totally displaced vWF binding and specifically bound to collagen. At saturation, 25 to 45 μg of SpIII bound per milligram of collagen. Scatchard plots demonstrated the presence of a single class of binding sites. SpIII was further digested with the same enzyme to generate SpI, a 52-kilodalton fragment from the C-terminal part of SpIII (residues 911 to 1,365). SpI induced a dose-dependent inhibition of both vWF and SpIII binding to collagen. A series of six monoclonal antibodies against SpIII that completely abolished vWF and SpIII interaction with collagen also bound to SpI. In conclusion, SpI extending between amino acid residues 911 and 1,365 of vWF contains a specific site that interacts with human type III collagen.

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HUMAN VON WILLEBRAND FACTOR (vWF) is a large plasmatic glycoprotein that participates at the site of vessel wall injury in platelet adhesion to subendothelium at high shear rates.[1,2] This function appears to occur by the bridging of platelets to subendothelium components through several sites on vWF, each specific for a distinct receptor. Three systems have been presently identified. Two involve glycoproteins of the platelet membrane,[3] ie, glycoprotein Ib (GPIb)[3-7] and the GPIIb/IIIa complex,[3,8-11] and require the presence of a specific agonist. The third binding site is located in the subendothelium, possibly on the collagen fibers. It has been established that native vWF spontaneously binds to collagen.[12-15]

In plasma, vWF appears as a set of multimers ranging from 0.5 or 1 to more than 15 × 10^6 daltons.[16-18] Each multimer results from the association of disulfide-linked identical subunits composed of 2,050 amino acids with a total molecular weight (mol wt) of 275 kilodaltons (kd).[19-21]

Several studies on the structure-function relationship of vWF have been performed, that are based on limited proteolytic degradation of the protein and the use of monoclonal antibodies (MoAbs) to vWF. These studies have allowed the separation and identification of several domains of vWF involved in its function(s).[22-32] Using *Staphylococcus aureus* V-8 protease (V-8 protease) digestion of vWF, we previously established that the enzyme acted by a primary cut (Glu 1,365 to Glu 1,366) in the vWF subunit.[21,27] This produced two dimeric fragments with a complementary sequence. The C-terminal fragment SpII has a mol wt of 215 kd unreduced and is composed of two identical chains of 110 kd (residues 1,366 to 2,050). This fragment contains the vWF domain interacting with platelet GPIIb/IIIa after thrombin stimulation.[28] The complementary N-terminal fragment SpIII has a mol wt of 320 kd unreduced and is composed of two 170-kd subunits (residues 1 to 1,365). SpIII specifically binds to platelet membrane GPIb in the presence of ristocetin[27-29] and retains the ability to promote platelet adhesion to purified collagen.[30]

Comparison of tryptic[23] and V-8 protease[28] digestion of vWF strongly suggested that a collagen binding domain was located in a central portion of the vWF subunit, between the GPIb and GPIIb/IIIa binding domains (amino acids 728 to 1,365).[31] Pareti et al[32] recently showed, however, that a collagen binding site was located between residues 449 and 728, the same 46-kd tryptic fragment that already contained a GPIb binding domain[25] and a heparin binding site.[26]

To further characterize the collagen binding domains of vWF, we now have investigated the ability of purified SpI, a central fragment of the vWF subunit that does not bind to ristocetin-treated platelets,[27] to interact with collagen. SpI results from a slow, secondary cut by V-8 protease between residues Glu 910 and Gly 911. It corresponds to the C-terminal end of SpIII and is released as a monomeric fragment with a mol wt of 45 kd unreduced and 52 kd reduced. We report here competitive inhibition studies of vWF and SpIII binding to collagen and direct analysis of the interaction between collagen and vWF or vWF fragments. We also used MoAbs to vWF previously characterized as potent inhibitors of vWF binding to collagen, GPIIb/IIIa, or GPIb[15,28] to establish that a functional site of the vWF subunit responsible for binding to collagen is located in the sequence of SpI between amino acid residues Gly 911 and Glu 1,365.

MATERIALS AND METHODS

vWF purification and digestion by V-8 protease. Human vWF was purified from factor VIII/vWF concentrates donated by the Centre National de Transfusion Sanguine, Paris, as described by Thorell and Blombäck.[33] The cryoprecipitate was gently dissolved in

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distilled water at 37°C and extensively dialyzed against 25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 2 mol/L glycine. The precipitate was centrifuged at 10,000 g for 20 minutes at 10°C. vWF was isolated from the supernatant by gel filtration on Sepharose CL-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as already described.[17] Purified vWF was concentrated by precipitation with 40% saturated ammonium sulfate and stored at 80°C. Purified vWF contained 200 to 230 vWF antigen U/mg of protein. It appeared as a set of ten to 11 bands by sodium dodecyl sulfate (SDS) agarose–polyacrylamide gel electrophoresis. The concentration of fibrinogen and fibronectin in vWF preparations was less than 0.1% as estimated by radioimmunoassay.

Proteolysis of vWF by V-8 protease (Miles Laboratories, Inc, Naperville, IL) and the purification of fragments SpI, -II, and -III were carried out as previously described.[27,28] SpI was concentrated by dialysis using UH 100/10 membranes (Schleicher and Schüll, Dassel, FRG).

Characteristics of the MoAbs to vWF. MoAbs to human vWF were produced and characterized as previously described.[34,35] All were $\gamma 1$, κ IgG fractions that were purified from ascites fluids as reported.[35] MoAbs were selected for their ability to specifically inhibit interaction of vWF with either platelets or collagen. MoAbs 311, 322, and H9 block the binding of vWF and SpIII to ristocetin-treated platelets.[15,28,35] MoAbs B200 through B205 inhibit the interaction of vWF with type I collagen.[15] Epitopes 311, 322, H9, and B200 to B205 were previously localized on SpIII.[28] MoAb 9 abolishes the binding of vWF[36] and SpII[28] to thrombin-activated platelets and recognizes an epitope on SpII.[28] In this study an unrelated MoAb to a mammary tumor glycoprotein was used as control IgG.

Radiolabeling. vWF and fragments SpI, SpII, and SpIII were labeled by the method of Fraker and Speck[37] with ^{125}I (Amersham International, Amersham, England) and Iodo-Gen (Pierce Chemical Co, Rockford, IL). Free iodide was separated by gel filtration on a Sephadex G-25 column (Pharmacia) or by extensive dialysis against Tyrode's buffer (130 mmol/L NaCl, 2.6 mmol/L KCl, 12 mmol/L $NaHCO_3$, 5.5 mmol/L glucose, pH 7.2). The specific radioactivity of vWF and SpI, -II, and -III varied from 0.18 to 4.5 μCi/μg of protein. Labeled vWF and fragments were kept at 4°C and used within 24 hours after labeling. Labeled preparations of IgG had a specific radioactivity of 8 to 10 μCi/μg. Nonfibrillar collagen was labeled with Bolton-Hunter reagent.[38] Excess labeled reagent was eliminated by extensive dialysis against 0.1 mol/L acetic acid.

Preparation of collagen fibrils. Lyophilized human nonfibrillar type III collagen was purchased from Sigma Chemical Co, St Louis). This preparation was about 65% pure as determined by hydroxyproline analysis[39] using the content of hydroxyproline in type III collagen previously reported by Miller and Gay.[40] It was about 95% pure when analyzed by SDS–5% polyacrylamide gel electrophoresis,[41] which indicated the absence of contaminating proteins.

Human type III collagen fibrils were prepared from 3-mL aliquots of nonfibrillar collagen dissolved at 1 mg/mL in 0.1 mol/L acetic acid by extensive dialysis for 48 hours at 4°C against 20 mmol/L Na_2HPO_4, pH 7.5.[42] The pellet of collagen fibrils was collected by centrifugation at 20,000 g for one hour at 4°C. It contained 95% to 97% of the starting material as determined in control experiments by radioactivity counting using ^{125}I-collagen. Finally the collagen fibrils were resuspended in 20 mmol/L Na_2HPO_4, pH 7.5, by stirring at 4°C for 16 hours. They were stored for up to 4 weeks at 4°C without apparent loss of activity. Denatured collagen was prepared just before use by heating aliquots of collagen fibrils at 80°C for one hour.

Binding of vWF, SpIII, and SpI to collagen. ^{125}I-labeled vWF or SpIII were centrifuged for three minutes at 10,000 g (Beckman Microfuge M12, Palo Alto, CA) immediately before use. Binding assays were performed in Tyrode's buffer containing 2% bovine serum albumin (BSA) (Tyrode's-BSA buffer) at 22°C in 1.5-mL conical polypropylene tubes. The final mixture (0.2 mL) contained 40 μL of a suspension of collagen fibrils (0.07 to 0.5 mg/mL), 20 μL of a solution of 1% NP40, 100 μL of serial dilutions of ^{125}I-ligand and 40 μL of Tyrode's-BSA buffer. The mixture was incubated for 45 minutes at 22°C. Duplicate aliquots (75 μL) were layered onto 300 μL of 25% sucrose in Tyrode's-BSA buffer in 400-μL conical polyethylene tubes and centrifuged for 15 minutes at 10,000 g. The tips of the tubes containing the pellet were cut with a scalpel, and associated radioactivity was counted in a 1260 multigamma II counter (LKB Instruments SA, Bromma, Sweden). Free ^{125}I-ligand was also measured in the supernatant for each experimental point. Nonspecific binding was estimated as the radioactivity bound to heat-treated collagen. Specific binding was defined as total minus nonspecific binding. Scatchard plots were generated from the specific binding curves. Binding parameters for vWF and SpIII were calculated from experimental values assuming one binding site per subunit with mol wts of 275 and 170 kd, respectively. As established in control experiments using labeled collagen, more than 94% of the collagen fibrils were sedimented in the pellet under our binding assay conditions.

The affinity of iodinated and unlabeled ligand (vWF or SpIII) was compared in the binding assay according to the method described by Pareti et al,[32] using a constant concentration of ligand composed of various proportions of labeled and unlabeled protein. For each ligand, no significant difference in the binding behavior was observed within the range of specific radioactivities used in the present study.

In some experiments, collagen-bound ^{125}I-ligands were washed with Tyrode's buffer and centrifuged for three minutes at 10,000 g. The supernatants were removed and the pellets dissolved for 30 minutes at 37°C in 100 μL of 6 mol/L urea, 1% SDS, and 125 mmol/L Tris-HCl buffer, pH 6.8. The suspensions were centrifuged, and supernatants containing 80% to 90% of the radioactive material were analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

Binding of ^{125}I-SpI to collagen was performed by using experimental mixtures prepared as described in the binding assay with the only difference being that a constant amount of 1 μg/mL (final concentration) of ^{125}I-ligand was incubated with various amounts (10 to 250 μg/mL final concentration) of collagen fibrils.

Inhibition of binding of ^{125}I-vWF or ^{125}I-SpIII (final concentration, 1 μg/mL) to collagen by unlabeled proteins was performed by using a modified binding assay in which a constant amount of ^{125}I-ligand in Tyrode's-BSA buffer premixed with serial dilutions of unlabeled vWF, SpIII, SpII, or SpI was added to the reaction mixture. Results were expressed by comparison with the specific binding of ^{125}I-ligand estimated in the absence of a competitor.

When the effect of purified MoAbs (IgG) to vWF was analyzed upon ^{125}I-vWF or ^{125}I-SpIII binding, increasing concentrations of IgG were preincubated for 30 minutes at 37°C with a constant amount of ^{125}I-ligand, and the mixture was centrifuged for three minutes at 10,000 g before addition to the other reagents. The supernatant (100 μL) was incubated with collagen (final concentrations, 1 μg/mL of ^{125}I-ligand, 100 μg/mL of collagen).

SDS-gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli[43] and Miller and Rhodes.[41] Vertical slab gels were run at 80 V (constant voltage) for 18 hours. SDS-agarose–polyacrylamide slab gel electrophoresis was performed as previously described.[20,28] Markers included low mol wt standards (Pharmacia), purified IgG (mol wt, 160 kd), fibronectin (mol wt, 440 kd), and IgM (mol wt, 900 kd). The gels were stained with Coomassie blue, dried, and autoradiographed at −80°C using Kodak X-Omat S films and X-Omatic cassettes (Eastman Kodak &

Co, Rochester, NY). Alternatively, the gels were blotted onto nitrocellulose paper (Schleicher and Schüll),[44] incubated with ^{125}I-labeled MoAb IgG, and extensively washed with 0.15 mol/L NaCl, and the reactive proteins were visualized by autoradiography.

RESULTS

Binding of vWF to human collagen. Preliminary experiments established that the vWF-collagen interaction was rapid and reached a maximum within 30 minutes and that no significant modification of the equilibrium occurred for 24 hours (data not shown).

When increasing concentrations of purified ^{125}I-vWF were used as the ligand, concentration-dependent and saturable binding to human fibrillar type III collagen (100 μg/mL) was observed. In the experiment described in Fig 1, the total amount of bound vWF increased from 3.5 to 43.6 μg/mg of collagen. Nonspecific binding linearly increased and reached 6.3 μg/mg of collagen at the highest vWF concentration. The binding parameters derived from Scatchard analysis of the specific binding curve indicated that two classes of binding sites were involved in vWF-collagen interactions (Fig 1). At saturation, the maximal binding of vWF was 43 μg/mg of collagen comprising 10 μg/mg with an apparent high affinity K_D of 1.8 μg/mL, and 33 μg/mg with an apparent low affinity K_D of 39 μg/mL. Similar results were obtained in two other experiments using lower concentrations of collagen (15 and 50 μg/mL) and different preparations of labeled vWF (Table 1).

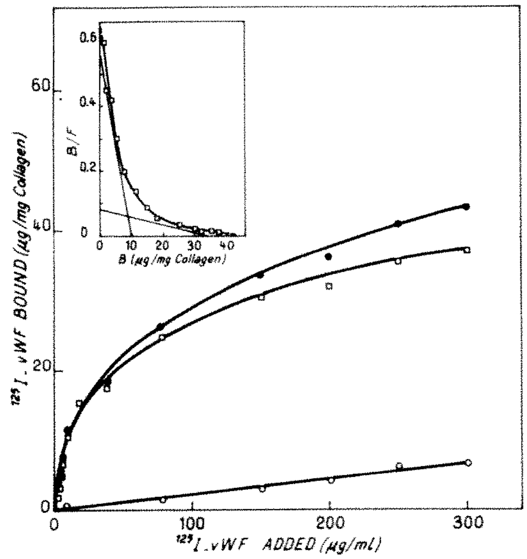

Fig 1. Binding of purified ^{125}I-vWF to collagen. Increasing amounts of ^{125}I-vWF (final concentrations, 1.2 to 300 μg/mL) were incubated at 22°C with a suspension of human type III collagen fibrils. After 45 minutes the collagen-associated radioactivity was separated from free ^{125}I-ligand by centrifugation (15 minutes, 10,000 g) as described in Materials and Methods. In this experiment, ^{125}I-vWF had a specific radioactivity of 0.9 × 10^6 cpm/μg, and the final concentration of collagen was 100 μg/mL. Specific binding (□) was estimated by subtracting nonspecific (O) from total binding (●). Nonspecific binding was measured by using heat-denatured collagen. The inset shows the Scatchard plot derived from the specific binding values.

Table 1. Parameters of Binding of ^{125}I-vWF to Human Fibrillar Type III Collagen

Experiment No.	Collagen (μg/mL)	Bound ^{125}I-vWF (10^{14} sites/mg Collagen) Total	High Affinity	Low Affinity	K_D (10^{-8} mol/L) K_H	K_L
1	15	0.83	0.15	0.68	0.33	10.7
2	50	1.1	0.22	0.88	1.0	21
3	100	0.94	0.22	0.72	0.65	14

Parameters were calculated from Scatchard analysis of the binding isotherms and assuming one binding site per vWF subunit with a mol wt of 275 kd.

Binding of SpIII to human collagen. Binding data obtained using 100 μg/mL of collagen and increasing concentrations of ^{125}I-SpIII (from 1.2 to 300 μg/mL) are shown in Fig 2. Total binding increased from 0.4 to 26.7 μg/mg of collagen. Nonspecific binding linearly increased and reached 4 μg bound/mg of collagen at the highest SpIII concentration. Scatchard analysis of the specific binding curve (Fig 2) indicated that saturation was obtained when 31 μg SpIII bound/mg of collagen and that a single class of sites existed for SpIII with an apparent K_D of 97 μg/mL. Similar results were observed in four separate experiments using different concentrations of collagen and various preparations of labeled SpIII (Table 2).

Inhibition of binding of vWF and SpIII to collagen by specific MoAbs to vWF. In these experiments selected MoAbs to vWF were tested to identify the domain on SpIII and vWF involved in binding to collagen. As illustrated on Fig 3, MoAbs 9 and 322 had no significant inhibitory effect

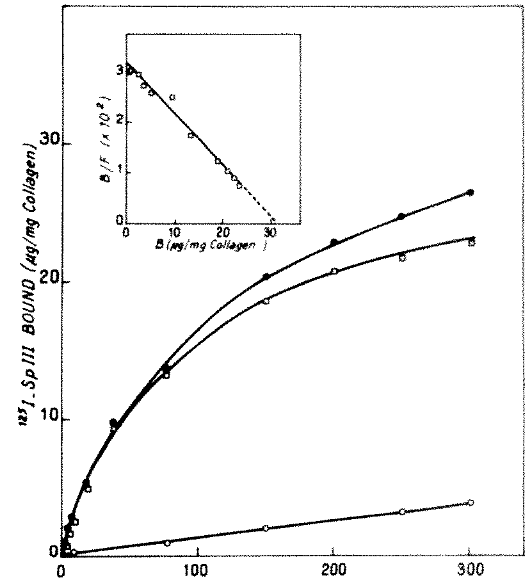

Fig 2. Binding of purified ^{125}I-SpIII to collagen. Increasing amounts of ^{125}I-SpIII (final concentrations, 1.2 to 300 μg/mL) were incubated with human fibrillar type III collagen at 22°C for 45 minutes. In this experiment, ^{125}I-SpIII had a specific radioactivity of 0.6 × 10^6 cpm/μg, and the final concentration of collagen was 100 μg/mL. Specific binding (□) was estimated as described in the legend of Fig 1. The inset shows the Scatchard plot derived from the specific binding values.

Table 2. Parameters of Binding of ^{125}I-SpIII to Human Fibrillar Type III Collagen

Experiment No.	Collagen (μg/mL)	Bound ^{125}I-SpIII (10^{14} Sites/mg Collagen)	K_D (10^{-8} mol/L)
1	15	1.1	35
2	20	1.6	38
3	50	0.9	59
4	100	1.1	57

Parameters were calculated from Scatchard analysis of the specific binding curve and assuming one binding site per SpIII subunit with a mol wt of 170 kd.

upon ^{125}I-vWF or ^{125}I-SpIII binding to collagen. Similar results were obtained when using MoAbs H9 and 311. In contrast, MoAbs B200 to B205, directed towards SpIII and known to inhibit ^{125}I-vWF binding to collagen, also decreased in a dose-dependent manner the binding of ^{125}I-SpIII to collagen. These results were confirmed in four separate experiments in duplicate.

Localization of a collagen binding domain on SpI by using competitive inhibition and direct binding. Purified SpI, -II, and -III fragments produced by selective V-8 protease digestion of vWF were compared with purified vWF for their ability to competitively inhibit binding of ^{125}I-vWF or SpIII to collagen (Fig 4). When ^{125}I-vWF was used as the ligand, complete inhibition of binding was observed with three of the competitors, ie, vWF, SpIII, and SpI. Half-maximum inhibition was reached with 1.5 μg/mL vWF, 105 μg/mL SpIII, and 150 μg/mL SpI, which corresponds to 5.5, 620, and 2,900 nmol/L, respectively. In contrast, even at 500 μg/ml, SpII was unable to compete for the binding of ^{125}I-vWF to collagen. Similar experiments using ^{125}I-SpIII as the ligand confirmed the presence of interactive sites of vWF with collagen on SpIII as well as on SpI (Fig 4). Binding of ^{125}I-SpIII was fully abolished by vWF and by SpIII. It was also almost completely inhibited at the highest concentration (300 μg/ml) of SpI used. Half-maximum inhibition induced by each competitor was reached with 1.0, 40, and 90 μg/mL, respectively, which corresponds to 3.6 nmol/L for vWF, 235 for SpIII, and 1,730 for SpI. SpII at concentrations up to 500 μg/mL did not modify the binding of ^{125}I-SpIII to collagen.

^{125}I-SpI was also tested for its capacity to bind directly to collagen. When using various concentrations (between 10 and 250 μg/mL) of collagen and a constant concentration of ^{125}I-ligand (1 μg/mL), a low but significant and reproducible specific binding of SpI to collagen occurred. The total binding linearly increased from 0.1 ± 0.05% to 2 ± 0.2% (mean ± SD, n = 5) of the added ^{125}I-SpI as compared with a

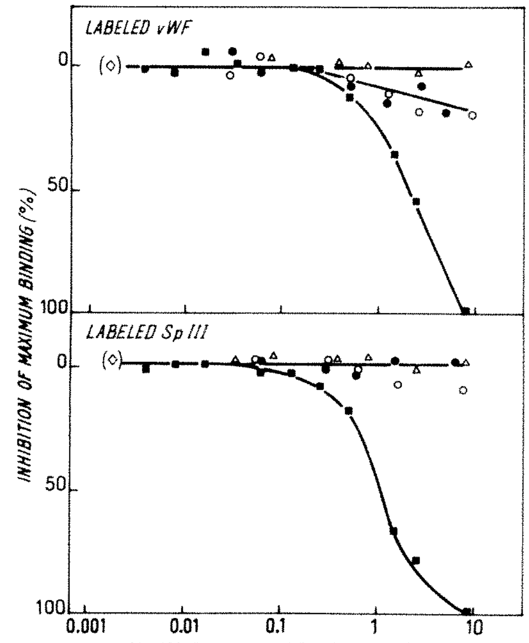

Fig 3. Effect of MoAbs to vWF upon binding of ^{125}I-vWF or SpIII to collagen. Preincubation of ^{125}I-ligand (1 μg/mL, final concentration) with increasing concentrations of MoAb IgG was performed as described in Materials and Methods with 100 μg/mL of human fibrillar type III collagen. In this experiment, the total count in 75-μL aliquots was ~710,000 cpm for ^{125}I-vWF and ~480,000 cpm for ^{125}I-SpIII. In the absence of IgG, total binding was 18% for vWF and 13% for SpIII. Nonspecific binding (3,000 and 1,000 cpm, respectively) did not vary with the concentration of IgG added, and it was subtracted from total binding to estimate specific binding (◊). Results are expressed as percentages of inhibition of specific binding estimated in the absence of IgG. ^{125}I-ligand was incubated with MoAb B203 (■), MoAb 9 (●), MoAb 322 (○), or control MoAb IgG (△).

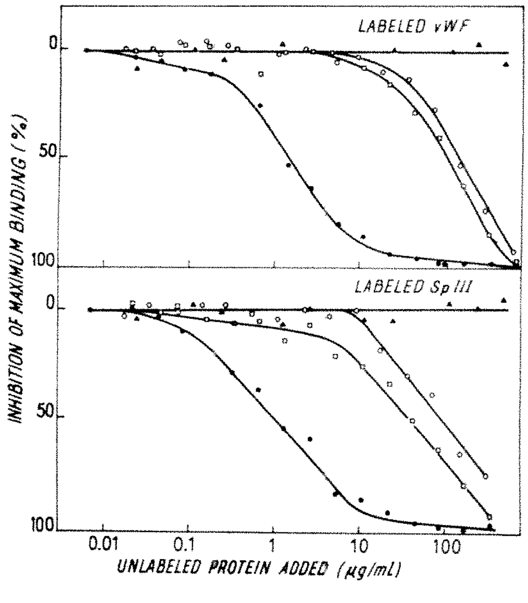

Fig 4. Inhibition of binding of ^{125}I-vWF and ^{125}I-SpIII (1 μg/mL, final concentration) to human collagen by unlabeled vWF or by fragments SpI, SpII, and SpIII. In this experiment, human fibrillar type III collagen (100 μg/mL, final concentration) was added at 22°C to a mixture of either ^{125}I-vWF (9.1×10^6 cpm/μg, top panel) or ^{125}I-SpIII (7×10^6 cpm/μg, bottom panel) with increasing concentrations of unlabeled protein. After 45 minutes, 75-μL aliquots of each sample that contained ~680,000 and ~520,000 cpm, respectively, were centrifuged in duplicate through 25% sucrose as described in Materials and Methods. In the absence of a competitor, the total binding was 23.6% of the applied radioactivity for ^{125}I-vWF and 11% for ^{125}I-SpIII. In both cases nonspecific binding was ~0.7% and did not vary with the concentrations of unlabeled protein added. Results are expressed as percentages of inhibition of specific binding estimated in the absence of competitor. ^{125}I-ligand (vWF or SpIII) was incubated with vWF (●), SpIII (□), SpI (○), or SpII (▲).

nonspecific binding linearly varying from 0.02 ± 0.02% to 0.4 ± 0.08%.

Localization of a collagen binding domain on SpI by using MoAbs to vWF. The presence of a collagen binding domain on purified SpI was confirmed by SDS–polyacrylamide gel electrophoresis followed by immunoblotting with labeled MoAbs to vWF (Fig 5). Purified SpI migrated as a single band with a mol wt of 45 kd (Fig 5, left lane). After blotting, SpI reacted with labeled MoAb B203 (Fig 5, right lane). Similar results were obtained when using the other MoAbs from the same series (B200 to B205) that are known to be specific for the collagen binding domain of vWF and SpIII. In contrast, epitopes 9 and 322 were not detectable on SpI (Fig 5).

DISCUSSION

Our present data, which were obtained using purified fragments SpI, SpII, and SpIII isolated after V-8 protease digestion of vWF, now provide direct evidence that a site specific for collagen is located in the central part of the vWF subunit on fragment SpI between residues 911 and 1,365.

The localization of collagen-interactive sites on SpIII, a dimer of the N-terminal end of vWF (residues 1 to 1,365) already known to bind to platelet GPIb,[27] was clearly established by showing specific saturable binding of this fragment to type III collagen. In addition, the competitive inhibition of ^{125}I-vWF binding by SpIII and of ^{125}I-SpIII binding by vWF strongly suggests that these two species share the same binding domains for collagen. These data are in agreement with previous results showing that SpIII may substitute for vWF in promoting platelet adhesion to collagen at high shear rates.[30] Conversely, SpII, a dimer of the C-terminal end of vWF extending from residues 1,366 to 2,050,[27] demonstrated no ability to interact with collagen by either direct binding assays or competitive inhibition studies. Accordingly, SpII has been shown to have no effect upon platelet adhesion to collagen even though it contains a specific binding domain for platelet GPIIb/IIIa.[28,30]

The localization of collagen-interactive sites on the vWF subunit relies upon the use of MoAbs to vWF B200 to B205, which are specific for SpIII and markedly inhibit vWF binding to type I[15] and type III collagen. Our data show that the occupancy of the corresponding epitopes on SpIII or on vWF produced in both cases a total inhibition of binding of the corresponding proteins to collagen. We showed as a control that MoAbs to SpIII that interfered with binding of SpIII and vWF to platelet GPIb (MoAbs 311, 322, H9) or MoAbs to SpII that blocked binding of SpII or vWF to GPIIb/IIIa (MoAb 9) had no significant effect upon the interaction of vWF or SpIII with collagen. A more precise localization of a specific domain for collagen on the vWF subunit came from results of previous experiments using V-8 protease or trypsin vWF degradation fragments[23,28] as well as specific MoAbs to vWF epitopes involved in binding to platelet GPIb, GPIIb/IIIa, or collagen. We had recently hypothesized that a domain interacting with collagen was located in the central portion of the vWF subunit between the N-terminal GPIb binding site and the C-terminal GPIIb/IIIa binding domain.[31] This hypothesis is now confirmed by our experiments using purified SpI. This monomeric fragment produced by secondary V-8 protease digestion of vWF corresponds to the C-terminal part of SpIII (residues 911 to 1,365) in the central part of the vWF subunit.[27] Our findings establish that SpI totally abolishes the interaction of ^{125}I-vWF and ^{125}I-SpIII with fibrillar human type III collagen. Similar results were observed when using equine type I collagen (not shown). In addition, our data demonstrate that MoAbs B200 to B205, which are known to block vWF binding to collagen and to bind to SpIII, also recognize epitopes located on SpI. We thus conclude that a domain of vWF on SpI accounts for vWF ability to interact with collagen. Our results are in apparent conflict with those recently shown by Pareti et al using tryptic digestion of vWF.[32] These authors showed that a proteolytic fragment of the vWF subunit (residues 449 to 728), which was able to bind to GPIb[25] and to heparin,[26] also contained a domain specific for binding to type I collagen.[32] More recently, using cyanogen bromide fragmentation of reduced and alkylated vWF, Roth et al[45] showed that two sequences, extending from residues 542 to 622 and 948 to 998, respectively, appeared to interfere with the binding of vWF to collagen.[21] Combination of these results thus strongly suggests the presence of at least two binding sites for collagen on each vWF subunit, one in the N-terminal portion and the other in the central part of the vWF subunit.

The relative importance of both domains in the function of native vWF remains to be clarified. Comparison of their affinity for collagen is speculative because vWF fragments isolated after enzymatic degradation may not clearly reflect the function of the corresponding sequences in a native conformation. In the present study, we observed that SpIII, which contains both functional sequences, exhibited a single type of binding site for collagen. These results strongly suggest that the two domains have a similar efficiency in binding. Other lines of evidence suggest that binding of vWF to collagen requires the presence of both sequences on the molecule and that the two sites do not act independently. We thus observed that purified SpI, which apparently contains a single domain, had a low affinity for type III collagen, thereby preventing an accurate estimation of its binding parameters. Nevertheless, this fragment kept the ability to totally inhibit the binding of species containing both active

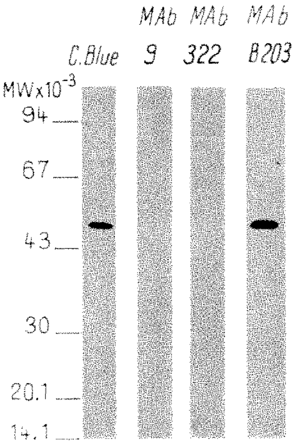

Fig 5. Localization of a vWF domain interacting with collagen by using specific MoAbs to vWF. After SDS–polyacrylamide (7.5%) gel electrophoresis, SpI was stained with Coomassie blue (C.Blue) or transfered onto nitrocellulose paper and displayed by using ^{125}I-IgG from MoAb 9, MoAb 322, or MoAb B203. The position of the mol wt markers is indicated on the left.

sequences like vWF or SpIII. Our hypothesis is further supported by comparing our data of inhibition of binding to collagen by MoAbs to vWF with those recently shown by Pareti et al.[32] These authors found that blocking one epitope between residues 449 and 728 totally inhibited the binding of vWF to collagen, whereas we observed that blocking a single epitope between residues 911 and 1,365 (SpI) also totally abolished binding of vWF, again suggesting that an interaction between both sites is required for binding of vWF to collagen.

The present study also clearly defines the parameters of vWF binding to human type III collagen. We established that its is specific and saturable. Analysis of the binding isotherms demonstrated a biphasic Scatchard plot suggesting the presence of at least two types of interactions. One possibility is the presence of two binding sites with distinct affinity on collagen. vWF subunits may either bind with a high (~1 vWF subunit per 100 to 140 collagen trimers) or a lower affinity (~1 vWF subunit per 20 collagen trimers). These values are consistent with those found by others[32,45] using different types of collagen. However, the two types of binding to collagen were only found under our experimental conditions using human type III collagen. An alternative model should be considered because of the multivalency of the vWF multimers. At low vWF concentrations, ie, an excess of binding receptors on collagen, one may assume that all the vWF subunits in one multimer are bound. Thus, as in the previous model, the mol wt of the ligand molecule would be that of the vWF subunit. The K_D value and number of receptors would be those previously determined for the high-affinity sites. In contrast, at saturating concentrations of vWF, one would expect that a bound multimer would be attached only through one subunit. The other subunits linked by disulfide bridges would be an accurate indication of the mass of vWF bound to collagen but would lead to an overestimation of the vWF subunit directly bound to the receptor. Thus a more accurate estimation of the maximal number of receptors on collagen would be reached by using the average mol wt of the vWF multimers previously established.[20] Using this assumption we found that at saturation only one vWF molecule bound per 130 to 172 collagen trimers. This is in close agreement with the number of vWF molecules bound to collagen trimers experimentally determined for the high-affinity binding sites. Further evidence to support this model is that the homogeneous SpIII molecule that contains the vWF sequences for the putative collagen binding receptors demonstrates only a single type of affinity by Scatchard analysis. Thus, these results suggest that a single type of vWF receptors exists on collagen and that the biphasic Scarchard plot is probably related to the heterogeneity of the multimer size of this large protein.

In conclusion, the present study demonstrates that a collagen interactive site exists in the central part of the vWF subunit, SpI, located from residues 911 to 1,365 in between a previously located N-terminal fragment binding to GPIb, heparin, and collagen[25-29,32,45,46] and a C-terminal fragment previously shown to bind to GPIIb/IIIa.[28] We do not yet know the relative importance of the two collagen binding domains in the function of native vWF. Such information would require a more precise determination of the effect of selected MoAbs upon collagen-vWF interactions.

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Expression of c-*abl* in Philadelphia-Positive Acute Myelogenous Leukemia

By Razelle Kurzrock, Mordechai Shtalrid, Moshe Talpaz, William S. Kloetzer, and Jordan U. Gutterman

The identical cytogenetic marker, t(9;22)(q34;q11) (Philadelphia [Ph] translocation), is found in approximately 90%, 20%, and 2% of adult patients with chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), and acute myelogenous leukemia (AML), respectively. In CML, the molecular events resulting from the Ph translocation include a break within the *bcr* locus on chromosome 22, transfer of the c-*abl* protooncogene from chromosome 9 to 22, and formation of an aberrant 210-kD *bcr-abl* fusion protein (p210$^{bcr\text{-}abl}$). Recently, the absence of *bcr* rearrangement and expression of a distinct aberrant 190-kd *abl* protein (p190$^{c\text{-}abl}$) has been described in Ph-positive ALL, with the suggestion that the two *abl* variants may be pathogenetically associated with myeloid *v* lymphoid leukemogenesis. Here we report that the genomic configuration and translation product of Ph-positive AML can be similar to that of Ph-positive ALL: the break at 22q11 may occur outside the 5.8 kb *bcr* region and result in expression of a 190-kD *abl* protein lacking these *bcr* sequences. Phosphokinase enzymatic activity, a fundamental property of p210$^{bcr\text{-}abl}$, was also associated with AML-derived p190$^{c\text{-}abl}$. Our current observations indicate that p190$^{c\text{-}abl}$ can be found in cells of lymphoid or myeloid lineage and is therefore unlikely to play a specific role in the development of lymphoid leukemias. Formation of p190$^{c\text{-}abl}$ instead of p210$^{bcr\text{-}abl}$ appears to be a characteristic of the acute rather than the chronic Ph-positive leukemic state.

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ONE OF THE MOST STRIKING examples of the association between a specific cytogenetic aberration and activation of a cellular protooncogene occurs in chronic myelogenous leukemia (CML). In 1960, Nowell and Hungerford[1] made the seminal discovery of a consistent chromosomal aberration—the Philadelphia (Ph) chromosome—in CML. The Ph chromosome is a shortened chromosome 22 resulting from a reciprocal translocation, t(9;22)(q34;q11).[2] Molecular analysis has revealed that the 22q11 breakpoint occurs within a breakpoint cluster region (*bcr*) spanning about 5.8 kilobase (kb).[3] Importantly, the c-*abl* protooncogene is transferred from its normal residence on chromosome 9 to chromosome 22[4] in juxtaposition to the 5′ *bcr* sequences that remain on the latter chromosome. The resulting fused *bcr-abl* gene expresses a novel 210-kD protein[5]; the normal c-*abl* counterpart is a 145-kD protein.[6] The aberrant 210-kD *bcr-abl* fusion protein (p210$^{bcr\text{-}abl}$) is active as a tyrosine phosphokinase,[5,6] a property of potential fundamental importance to CML because this enzymatic activity has been associated with the ability of several oncogenes and growth factors to exert their effects. Rearrangement of *bcr* and expression of a *bcr-abl* message occur in virtually all patients with CML[7] but not in patients with other hematologic disorders, strongly implicating these molecular events in the pathogenesis of CML.

Approximately 20% of adult patients with acute lymphoblastic leukemia (ALL) also carry the t(9;22)(q34;q11) anomaly.[8] Several investigators have shown molecular heterogeneity of chromosome 22 breakpoints in Ph-positive ALL[9-11]; at least one half of reported patients do not have rearrangement within the 5.8-kb *bcr* locus. The break at 22q11 may occur between the *bcr* and C_λ region of the immunoglobulin light-chain cluster gene.[10] Recently, our laboratory[12] and others[13,14] have demonstrated a novel aberrant 190-kD c-*abl* protein (p190$^{c\text{-}abl}$) in *bcr* rearrangement–negative, Ph-positive ALL; p210$^{bcr\text{-}abl}$ was not found. Presumably, p190$^{c\text{-}abl}$ results from recombination of c-*abl* with a DNA segment upstream of the 5.8 kb *bcr* locus. Based on these results, it appeared that subtle alterations in genomic configuration relative to the *bcr* region may correlate with distinct disease phenotypes. A question that arose is whether the generation of p190$^{c\text{-}abl}$ *v* p210$^{bcr\text{-}abl}$ is associated with the development of a lyphoid as opposed to myeloid phenotype or, alternatively, of an acute rather than a chronic leukemic process. One approach to dissecting these possibilities was to study samples from patients with a rare leukemia subtype—Ph-positive acute myelogenous leukemia (AML). In this paper, we demonstrate absence of *bcr* rearrangement in a Ph-positive AML patient and expression of a 190-kD c-*abl* protein indistinguishable from that found in *bcr* rearrangement–negative, Ph-positive ALL patients. Our results indicate that p190$^{c\text{-}abl}$ is not specific for the lymphoid phenotype but instead may be important in the manifestation of an acute rather than chronic Ph-positive leukemic process.

PATIENT AND METHODS

Case report. In May 1986, patient V1 presented with Ph-positive AML. Her past medical history included a diagnosis of carcinoma of the right breast in 1965. She underwent mastectomy and postoperative chest wall irradiation (5,000 rad). In 1970, a left breast carcinoma was found and subsequently removed by mastectomy. She returned to M.D. Anderson Hospital and Tumor Institute every 4 to 6 months for follow-up evaluation. As part of each visit, a CBC was done. In January 1986, the blood counts were normal: WBC count, $6 \times 10^3/\mu L$ with a normal differential count; hemoglobin, 12.7 g/dL; platelet count, $226 \times 10^3/\mu L$. At the time of her routine return appointment in May 1986, she felt well. There was no evidence of recurrent breast cancer. Surprisingly, laboratory tests revealed a grossly elevated WBC count of $179 \times 10^3/\mu L$ with 94% blasts. Her hemoglobin content was 8.1 g/dL; platelet count, $28 \times 10^3/\mu L$; and leukocyte alkaline phosphatase score, 5% (normal

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range, 40% to 225%). A bone marrow aspirate and biopsy specimen revealed a cellularity of 95% with 84% blasts. There was no peripheral blood or bone marrow basophilia. The majority of the bone marrow cells stained positive for myeloperoxidase, consistent with a diagnosis of AML. Reactivity with monoclonal antibody against the differentiation marker My4 was about 50% positive. Cell surface markers for OKT11 (T cell surface marker) and surface immunoglobulin (B cell marker) were negative. These results as well as morphological analysis of the bone marrow by light and electron microscopy indicated a diagnosis of M4 (myelomonocytic) subtype of AML (French-American-British classification). Karyotype analysis (Giemsa-stained chromosomes) revealed a hyperdiploid (51 to 87 chromosomes) Ph-positive clone in 25 of 25 (100%) bone marrow metaphases. The patient was treated with high-dose cytosine arabinoside and achieved complete hematologic remission after one course. However, the aberrant cytogenetic clone persisted in 10% of the cells. The patient remains in hematologic remission at 6+ months. Blood for molecular studies was obtained immediately before initiating chemotherapy and stored at −70°C until analysis. Informed consent, in accordance with institutional guidelines, was obtained for chemotherapeutic procedures and for drawing blood.

Antisera. Rabbit antisera were made against the following peptides: the predicted hydrophilic v-*abl* sequence DEVEKELGKRGTRGG-C (*abl*389-403); the predicted high-term potential domain of c-*abl* (Chou-Fasman predictive model for protien secondary structure, KENLLAGPSENDPN-C (*abl*-3); the region DDESPGLYGFLNV-C (*bcr*3) within *bcr* exon 3; and the NH$_2$-terminal peptide sequence 1 to 17 derived from the Heisterkamp et al[15] *bcr* sequence (*bcr*1). The methodology has been previously reported in detail by Kloetzer et al.[5]

DNA analysis. DNA was prepared by cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. DNA was analyzed by restriction enzyme digestion, Southern blotting, and hybridization of filters to nick-translated probes. The methodology was identical to that previously used in our laboratory.[12] A 3′, 1.2-kb *bcr* (*Hin*dIII-*Bgl*II) plasmid probe (Oncogene Sciences, Inc, Mineola, NY)[3] and a 5′, 0.45-kb (*Eco*RI/*Pst*I fragment) *bcr* cDNA probe (donated by E. Canaani)[16] were used.

Immune complex kinase assays. Proteins from blood samples were analyzed by the immune complex kinase assay (ICKA) under identical conditions to those previously described by our laboratory.[17] K562 cells (CML erythroid blast crisis cell line) were assayed as controls.

RESULTS

Analysis of leukemia cells for bcr *rearrangement.* To determine whether *bcr* rearrangement was present in patient samples, we performed Southern blotting and hybridization with a 3′, 1.2-kb (*Hin*dIII-*Bgl*II) genomic *bcr* probe. Hybridization of blots of *Bam*HI-, *Bgl*II, *Hin*dIII-, and *Eco*RI-digested DNA did not reveal rearrangement within *bcr* in Ph-positive AML patient V1 (Fig 1, even lanes). In contrast, Fig 1 (odd lanes) illustrates rearranged bands in *Bam*HI-, *Bgl*II, and *Eco*RI-digested, Ph-positive, CML-derived DNA. Because a DNA deletion in the segment encompassing the sequence of the 3′ *bcr* probe could result in a false-negative result for *bcr* rearrangement in AML patient V1,[18] we probed the filters with a 5′ (*Eco*RI/*Pst*I fragment) *bcr* probe.[16] Rearrangement in *bcr* was not observed (data not shown).

Analysis of c-abl *protein expression.* To study c-*abl* protein expression, we perform ICKAs. This assay can detect *abl*-related proteins because they are enzymatically active as phosphokinases. Consistent with our previously published results,[5,12] anti–*abl*389-403 serum and anti-*bcr*3 serum detected p210$^{bcr\text{-}abl}$ in extracts from the K562 CML blast crisis cell line (Fig 2A, lanes 1 and 3). To demonstrate that p210 is *bcr-abl* specific, we preincubated each of the antisera with the peptide against which they were made (Fig 2A, lanes 2 and 4). This procedure results in blocking of antisera-specific bands but not nonspecific background phosphorylation. To date, we have studied fresh samples from 16 Ph-positive CML blast crisis patients (myeloid blast crisis, ten patients; lymphoid, five patients; undifferentiated, one patient); all patients produced p210^{bcr} (Fig 2A, lanes 5 and 6; data not shown).[19] We have recently demonstrated that a Ph-positive ALL patient without *bcr* rearrangement expresses a distinct aberrant p190$^{c\text{-}abl}$ rather than p210$^{bcr\text{-}abl}$.[12] The mRNA encoding p190$^{c\text{-}abl}$ lacks at least some *bcr* sequences and is smaller (7.4 kb) than the 8.5-kb CML-derived *bcr-abl* transcript.[12] As shown in Fig 2A (lanes 7 and 9), *bcr* rearrangement–negative AML patients VI does not produce p210$^{bcr\text{-}abl}$ despite the presence of the Ph translocation; however, a 190-kD *abl*-related protein is detected by two anti-*abl* sera (Fig 2A, lane 7, and Fig 2B, lane 1). Figure 2B (lanes 1 and 3) demonstrates that the *abl*-related product of AML patient V1 comigrates with the p190$^{c\text{-}abl}$ of a Ph-positive *bcr* rearrangement–negative ALL patient.[12] Similar to the situation in ALL,[12] AML-derived p190-^{abl} is not detected by anti-*bcr*3 serum directed against *bcr* exon 3 (Fig 2A, lanes 9 and 10) or by anti-*bcr*l serum directed against the most 5′ portion of the Heisterkamp *bcr* sequences[15] (data not shown).

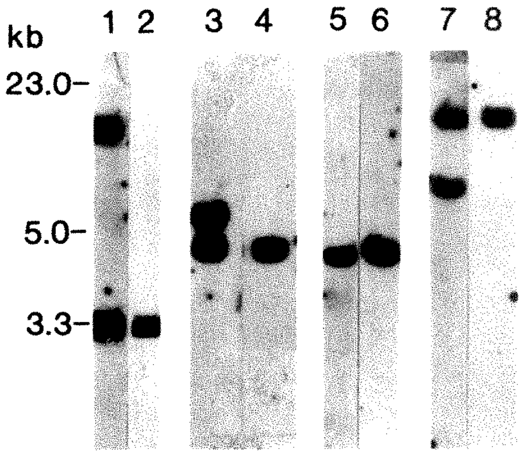

Fig 1. Southern blots of DNA extracted from Ph-positive leukemia patients and hybridized with a 3′, 1.2-kb (*Hin*dIII/*Bgl*II) genomic *bcr* probe (Oncogene Sciences). Restriction enzymes used were *Bam*HI (lanes 1 and 2), *Bgl*II (lanes 3 and 4), *Hin*dIII (lanes 5 and 6), and *Eco*RI (lanes 7 and 8). Odd lanes, Ph-positive CML patient; even lanes, Ph-positive AML patient VI.

A 190,000–molecular weight (mol wt) band is also observed in K562 cells (Fig 2A, lanes 1 and 3). This band may represent a second translational product of *bcr-abl* or a proteolytic fragment of p210$^{bcr\text{-}abl}$. The p190$^{c\text{-}abl}$ of AML patient V1 is distinct from the 190,000–mol wt band of Ph-positive CML cells because anti-*bcr* serum clearly recog-

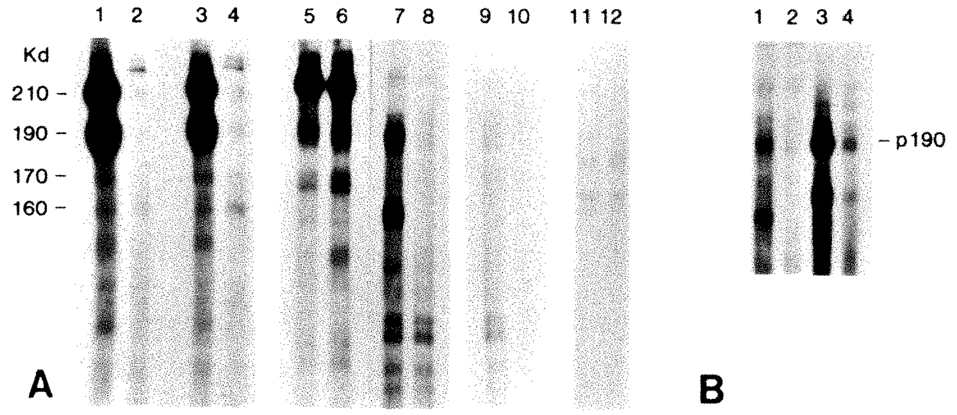

Fig 2. ICKAs. (A) Cells used were as follows: lanes 1 to 4, K562 cells (CML blast crisis cell line); lane 5, CML lymphoid blast crisis patient; lane 6, CML myeloid blast crisis patient; lanes 7 to 10, Ph-positive, *bcr* rearrangement–negative AML patient V1; lanes 11 and 12, Ph-negative AML patient. Antisera used were as follows: lanes 1, 2, 7, 8, 11, and 12, anti-*abl*389-403; lane 5, anti-*abl*3; lanes 3, 4, 6, 9, and 10, anti-*bcr*3. Blocking and antisera with cognate peptide was performed in lanes 2, 4, 8, 10, and 12. (B) Cells used were as follows: lanes 1 and 2, Ph-positive, *bcr* rearrangement–negative AML patient V1; lanes 3 and 4, Ph-positive, *bcr* rearrangement–negative ALL patient. The antiserum used was anti-*abl*3 in all lanes. Blocking of antisera with cognate peptide was performed in lanes 2 and 4.

nizes the latter protein (Fig 2A, lanes 3 and 6). Of course, because of their similar mol wts, we cannot rule out the possibility that CML patients produce $p190^{c\text{-}abl}$ in addition to $p190^{bcr\text{-}abl}$. Similarly, the 170,000–mol wt band in Ph-positive ALL cells (Fig 2B, lane 3) and the 160,000–mol wt protein in Ph-positive AML patient V1 (Fig 2B, lane 1) may also represent proteolytic fragments or additional translational modifications of the *abl* product.

DISCUSSION

The end stage of CML, termed blast crisis, strongly resembles Ph-positive acute leukemia: the leukemic cells lose their capacity for terminal differentiation, and similar clinical characteristics and laboratory parameters are observed.[20] Further, occasional chemotherapy-induced transition of Ph-positive ALL and AML to CML is well documented.[21] Based on these observations, it has been postulated that the disease of some patients presenting with Ph-positive acute leukemia actually represents the blast transformation stage of CML, which escaped diagnosis during its early, often asymptomatic chronic phase. Even so, it seems likely that at least some ALL and AML patients with t(9;22) have a bona fide acute presentation. Because Ph-positive acute leukemia and CML are similar but retain some distinct features, it may be that related but not identical molecular events are involved in their pathogenesis.

Interestingly, some ALL patients carrying a t(9;22), which is cytogenetically indistinguishable from that found in CML, do not have *bcr* rearrangement.[9-11] Our work[12] as well as that of other groups[13,14] has revealed a novel c-*abl* protein—$p190^{c\text{-}abl}$—in Ph-positive, *bcr* rearrangement–negative ALL patients; $p210^{bcr\text{-}abl}$ is not expressed. In the current report, we demonstrate that the Ph tranlocation in AML can also be associated with a 22q11 break outside of the *bcr* locus and production of $p190^{c\text{-}abl}$ without an accompanying $p210^{bcr\text{-}abl}$. Our data suggest that $p190^{c\text{-}abl}$ differs from $p210^{bcr\text{-}abl}$ by virtue of the absence of *bcr* sequences in the N-terminal of the former protein. Similar to the situation in ALL,[12] AML-derived $p190^{c\text{-}abl}$ is enzymatically active as a protein kinase, an observation consistent with a direct role for this aberrant protein in the malignant process. A literature review (Table 1) revealed that about 95% of Ph-positive CML patients (including at least 20 blast crisis patients)[22] but fewer than 50% of Ph-positive acute leukemia patients have *bcr* rearrangement. Further, $p210^{bcr\text{-}abl}$ appears to be expressed in all chronic[19] and acute leukemia patients[12] with *bcr* rearrangement. Although the two molecular subgroups of Ph-positive acute leukemia have similar clinical and morphological features,[10,11] their distinct genomic configurations may have biologic relevance. The presence of *bcr* rearrangement makes Ph-positive acute leukemia virtually indistinguishable from CML blast crisis[20] and suggests a single disease state, with the acute leukemia representing the blast transformation phase of subclinical CML. Conversely, Ph-positive *bcr* rearrangement–negative ALL and AML may reflect true de novo acute leukemia as evidenced by the following: (*a*) *bcr* rearrangement negativity is almost never found in CML and (*b*) $p190^{c\text{-}abl}$ without $p210^{bcr\text{-}abl}$ appears to be associated exclusively with the the acute leukemia phenotype. This concept is supported by our current observations because a preceding undetected CML state can be ruled out in our Ph-positive, *bcr* rearrangement–negative AML patient because of the fortuitous availability of normal blood counts obtained 4 months before diagnosis. Analysis of additional patients will be needed to strengthen the generality of these concepts. However, our data suggest that the presence or absence of proximal *bcr* sequences attached to c-*abl* may modulate this gene's tumorigenic potential for chronic *v* acute leukemogenesis.

Despite the presence of $p190^{c\text{-}abl}$ instead $p210^{bcr\text{-}abl}$, the subgroup of *bcr* rearrangement–negative, Ph-positive acute leukemia patients bears a close clinical resemblance to *bcr* rearrangement–positive, Ph-positive acute leukemia patients,[10,11] and as mentioned earlier, both subgroups resemble CML blast crisis.[20] The molecular events leading to the inevitable malignant evolution of CML from a chronic to an acute leukemia phenotype remain largely unexplored; however, because *bcr* rearrangement is found in the early benign

Table 1. Summary of Studies Analyzing Ph-Positive Leukemia Patients for *bcr* Rearrangement

Type of Leukemia	Source	No. of Patients Analyzed	No. of Patients With *bcr* Rearrangement (%)
CML	Groffen et al[3]	17	17
	Popenoe et al[18]	14	10
	Collins[23]	20	20
	Shtalrid et al[22]	72	70
Totals		123	117 (95)
ALL	Rodenhuis et al[9]	2	0
	Erikson et al[10]	5	2
	de Klein et al[11]	14	9
	Kurzrock et al[12,24]	6	2
	Clark et al[13]	4	0
	Chan et al[14]	4	1
Totals		35	14 (40)
AML	Erikson et al[10]	1	1
	Kurzrock et al (current report)	1	0
Totals		2	1 (50)

phase, it seems likely that superimposed secondary genetic events are required. If expression of p190$^{c\text{-}abl}$ without p210$^{bcr\text{-}abl}$ is associated with an acute leukemic state remarkably similar to CML blast crisis, is it possible that the production of p190$^{c\text{-}abl}$ in addition to p210$^{bcr\text{-}abl}$ occurs during the course of CML and plays a role in blast transformation? Further analysis of c-*abl* expression in the nosological spectrum of Ph-positive hematologic malignancies is in progress and should help elucidate the role of molecular variants of this gene on leukemogenesis.

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Fibrin Monomer Induces Binding of Endogenous Platelet von Willebrand Factor to the Glycocalicin Portion of Platelet Glycoprotein IB

By Robert I. Parker and Harvey R. Gralnick

This study demonstrates that when platelets are stimulated by thrombin in the presence of low concentrations of purified human fibrinogen (10 to 20 μg/mL, final concentration) binding of released platelet von Willebrand factor (plt-vWF) to the platelet membrane is enhanced. This effect appears to be mediated by fibrin monomer produced by the action of thrombin on the fibrinogen in the incubation suspension. When fibrin polymerization is inhibited, the binding of released plt-vWF to the platelets is markedly increased. This enhanced binding is dependent on platelet glycoprotein Ib (GPIb) as shown by a decreased response with Bernard-Soulier platelets and inhibition by both monoclonal and polyclonal antibodies against glycocalicin. The binding of fibrin to thrombin-activated platelets preincubated with monoclonal antibody against GPIIb/IIIa is increased when the predominant form of fibrin is fibrin monomer. The fibrin binding is also decreased in the presence of antibody against glycocalicin. Our data demonstrate that fibrin monomer facilitates plt-vWF binding to the glycocalicin portion of platelet GPIb on thrombin-stimulated platelets and that binding of fibrin monomer to glycocalicin is necessary for this response to occur.

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PLATELET von Willebrand factor (plt-vWF) is a multimeric glycoprotein localized primarily within platelet α-granules, with a small amount being expressed on the surface of resting platelets.[1-3] Surface expression of plt-vWF markedly increases after thrombin stimulation of platelets by the binding of released plt-vWF to the platelet surface.[3-5] The major platelet receptor involved in the binding of plt-vWF to activated platelets is the glycoprotein IIb/IIIa (GPIIb/IIIa) complex.[6] The thrombin-induced binding of plt-vWF can be inhibited by purified human fibrinogen (≥40 μg/mL) or by a monoclonal antibody directed against the fibrinogen and vWF binding sites on the GPIIb/IIIa complex. In contrast, a monoclonal antibody directed against the ristocetin-induced plasma vWF binding site (GPIb) has no effect on the thrombin-induced plt-vWF binding.[6]

The platelet GPIIb/IIIa complex also binds plasma vWF and fibrinogen when platelets are stimulated by adenosine diphosphate (ADP) or thrombin.[7-10] In addition to binding fibrinogen, thrombin-activated platelets also bind fibrin.[11] Recently, fibrin monomer stabilized on acrylonitrile beads has been shown to bind both plasma vWF and glycocalicin, a water-soluble proteolytic product of GPIb.[12]

In the current study, we demonstrate that the presence of fibrin monomer increases the thrombin-induced binding of endogenous plt-vWF to intact human platelets. The enhanced plt-vWF binding occurs on the glycocalicin portion of GPIb, and it requires the binding of fibrin monomer to the glycocalicin portion of GPIb.

MATERIALS AND METHODS

Platelets were isolated from fresh citrate/EDTA (13 mmol/L sodium citrate, 1mmol/L EDTA)-anticoagulated normal human whole blood by centrifugation of platelet-rich plasma through a discontinuous arabinogalactan (Stractan, Champion International, Tacoma, WA) gradient as previously described.[13] The gradient was sliced at the interface, and the platelets were aspirated and then diluted to a final concentration of 200,000/μL in Tyrode's buffer containing 5 mmol/L $CaCl_2$.

Surface-bound plt-vWF was determined by measuring the binding of a ^{125}I-labeled polyclonal affinity-purified rabbit antihuman vWF antibody to thrombin-stimulated platelets as previously described.[3,6] Briefly, 200-μL aliquots of platelets were incubated with radiolabeled anti-vWF antibody (final concentration, 3.75 μg/mL; 4.8×10^3 cpm/μg) at 37°C for 90 minutes. Various concentrations of purified human firbrinogen (10 to 80 μg/mL, final concentration), bovine serum albumin (BSA), or vWF-free purified human fibronectin (Sigma Chemical Co, St Louis) were included in the incubations. The platelet incubations were carried out in the following fashion: into each polypropylene vial was placed the radiolabeled ligand (anti-vWF or fibrinogen) and/or other proteins (fibrinogen, BSA, fibronectin), and thrombin was then placed along the inside wall of the vial. The amount of thrombin used produced a final concentration of 0.5 U/mL. Platelets were then added to the vial through the thrombin bubble. Fifteen seconds after the addition of the platelets, the thrombin was neutralized by hirudin (grade IV, Sigma; 20 U/mL, final concentration) to prevent fibrin clot formation, and the platelet suspensions were then incubated for 90 minutes at 37°C. In other studies, platelets were activated by calf skin collagen (226 μg/mL, final concentration, Cooper Biologic, Malvern, PA) At the completion of the incubation, bound ligand was separated from free ligand by centrifugation (9000 g for four minutes).

Fibrinogen was purified from lyophylized human-fibrinogen (Kabi grade L, Kabi Diagnostics, Stockholm) by passage over a diethyl aminoethyl (DEAE)-Sephacel column as previously described.[6] Fibrinogen prepared by this method was free of vWF and fibronectin as measured by radioimmunoassay and immunoprecipitation assay, respectively. In some experiments, platelets were preincubated with a saturating amount of a monoclonal antibody directed against the GPIb ristocetin-dependent binding site for plasma vWF (6D1) or against the GPIIb/IIIa binding site of fibrinogen and vWF (10E5). These monoclonal antibodies, provided by Dr B. Coller, State University of New York at Stony Brook, have been shown to have no effect on thrombin-induced platelet shape change or aggregation[8,14] or on thrombin binding to platelets.[15] In other experiments, a polyclonal antiserum monospecific for platelet glycocalicin was included in the incubation suspensions.[16] Because this antiserum inhibits thrombin binding to platelets,[15] it was added

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to the platelet suspensions (10%, vol/vol) after the thrombin had been neutralized by hirudin. The incubations were then continued for 90 minutes at 37°C. In some experiments, fibrin polymerization and fibrinogen binding to platelets were prevented by inclusion of the Gly-Pro-Arg-Pro (GPRP) tetrapeptide (Sigma) in the incubation (final concentration, 0.70 mmol/L), and in other experiments, fibrin polymerization was prevented by inclusion of purified bovine fibrinogen fragment D (final concentration, 1 μmol/L).

Platelets from two individuals with Bernard-Soulier syndrome were also studied. Platelets were isolated from citrate/EDTA-anticoagulated fresh whole blood by allowing the RBCs to settle by gravity for three hours at room temperature. Platelet-rich plasma was removed and centrifuged through a Stractan gradient as described earlier.

Fibrin(ogen) binding to activated platelets was measured by incubating 200-μL aliquots of platelets (200,000/μL) with ^{125}I-labeled, purified fibrinogen (7.2 × 10^4 cpm/μg) and α-thrombin or collagen under experimental conditions similar to those used to measure plt-vWF binding. The two exceptions were (*a*) the anti-vWF antibody was omitted and (*b*) the platelets were preincubated with monoclonal antibody 10E5 (20 μg/mL; 15 minutes at 22°C) to prevent binding of fibrinogen to GPIIb/IIIa. Bound fibrinogen was determined by measuring the radioactivity remaining with the platelet pellet. Additional experiments were performed to measure the binding of radiolabeled, purified fibrin(ogen) to platelets incubated with plasma vWF purified from cryoprecipitate (final concentration, 1 μg/mL) and ristocetin (ristocetin sulfate-A, Lenau and Co, Copenhagen; final concentration, 0.6 mg/mL).

The amount of fibrinogen cleaved to fibrin monomer after thrombin treatment was measured as free fibrinopeptide-A (FP-A) (Mallincrodt, St Louis). Purified fibrinogen was incubated with platelets or buffer and exposed to thrombin or collagen in a fashion identical to that described for the plt-vWF binding experiments. The supernatant was removed and frozen at −70°C until assayed for FP-A.

Fibrin monomer polymerization was determined spectrophotometrically by measuring the light absorbance of a suspension of fibrin(ogen) at 350 nm.[17] Purified fibrinogen at various concentrations (10 to 80 μg/mL) in Tyrode's buffer (pH 7.1) was cleaved by the addition of α-thrombin (0.5 U/mL) in either the presence or absence of the inhibitors of fibrin polymerization (GPRP tetrapeptide [0.70 mmol/L], bovine fibrinogen fragment D [1 μmol/L]). Hirudin (20 U/mL) was added 15 seconds after the addition of thrombin to quench the reaction, and from that point fibrin polymerization was followed by measuring the increase in light absorbance at 350-nm wavelength. Maximal fibrin polymerization occurred by five minutes, with no appreciable increase in fibrin polymerization even when the incubations were continued for 90 minutes. The ratio of change in light absorbance at 350 nm for fibrinogen with GPRP or fragment D (at five minutes) to change in light absorbance at 350 nm for fibrinogen without these peptides (at five minutes) corresponds to the fraction of fibrin polymerized. Zero percent polymerization is determined by the increase in 350 nm light absorbance for native fibrinogen without added thrombin, whereas 100% polymerization is determined by the increase in 350-nm light absorbance for fibrinogen cleaved by thrombin in the absence of the inhibitors of fibrin monomer polymerization. The measured amount of FP-A produced relative to the theoretical maximum amount of FP-A production at a given concentration of fibrinogen was used to determine the actual percentage of fibrinogen cleaved to fibrin monomer at that concentration of fibrinogen. Using this value and the percentage of fibrin polymer produced, as observed spectrophotometrically, we estimated the relative amounts of fibrin monomer and fibrin polymer produced under the various experimental conditions.

Thrombin was the kind gift of Dr J. Fenton (NY State, Department of Health, Albany). Bovine fibrinogen fragment D was provided by Dr E. Mihalyi (National Institutes of Health, Bethesda, MD), and the antiglycocalicin antiserum was obtained from Dr N. Solum (University of Oslo). All buffers were made up with distilled, deionized water (<10^{-6} mol/L Ca^{++}), and all chemicals were of reagent grade.

RESULTS

Figure 1 presents the results of plt-vWF binding to thrombin-stimulated platelets in the presence of increasing concen-

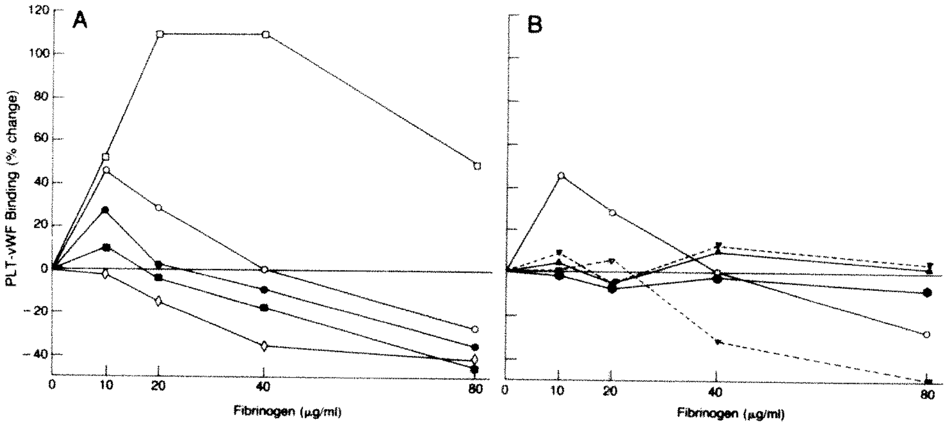

Fig 1. Percent change in plt-vWF surface expression on stimulated platelets in the presence of increasing concentrations of fibrinogen. Plt-vWF binding was determined by the binding of a radiolabeled anti-vWF antibody to platelets (see Materials and Methods). Results represent the means of five to six determinations and are expressed as percent change over control (no added fibrinogen). Aliquots of platelets (200 μL at 200,000/μL) were activated by α-thrombin (0.5 U/mL) or collagen (226 μg/mL). (A) Thrombin-stimulated platelets in the presence of increasing concentrations of fibrinogen and buffer (○), 1 μmol/L fibrinogen fragment D (□), 20 μg/mL 6D1 (●), 10% (vol/vol) α-GC antibody (■), and Bernard-Soulier syndrome platelets (◊). (B) Thrombin-stimulated platelets (○), thrombin-stimulated platelets with 0.70 mmol/L GPRP (▲), and collagen-stimulated platelets (●) in the presence of increasing concentrations of fibrinogen. The broken lines display the results obtained with thrombin-stimulated platelets incubated with increasing concentrations of fibronectin (▽) or BSA (▼). In these experiments, each protein was used in molar concentrations equivalent to those of fibrinogen. Nonspecific binding of the radiolabeled anti-vWF antibody in all experiments was typically less than 4%, and SEM for each point was typically less than 10% and usually less than 5%.

trations of fibrinogen. These data are presented as percent change in plt-vWF binding as compared with control platelets, ie, platelets stimulated by thrombin in the absence of fibrinogen or other plasma proteins. In these experiments fibrin(ogen) at concentrations of 10 and 20 μg/mL enhanced plt-vWF binding to normal thrombin-stimulated platelets, whereas higher concentrations of fibrinogen inhibited plt-vWF binding. A monoclonal antibody known to react with glycocalicin and to block ristocetin-induced binding of plasma-vWF to platelets (6D1) or a polyclonal antiserum monospecific for platelet glycocalicin (α-GC) reduced the fibrin enhancement of plt-vWF binding. The fibrin enhancement of surface bound vWF was not observed in the presence of the GPRP tetrapeptide (which inhibits fibrin polymerization and fibrin binding to platelets) or when Bernard-Soulier platelets were tested. Fibrin-enhanced binding of plt-vWF to the platelet surface was increased, however, when fibrin polymerization was prevented by the inclusion of 1 μmol/L bovine fibrinogen fragment D in the incubation suspensions (Fig 1A).

Fibrinogen had no effect on the surface vWF expression of resting platelets (data not shown) or on platelets stimulated by collagen (Fig 1B). Neither bovine fibrinogen fragment D nor the GPRP tetrapeptide had an effect on plt-vWF binding to thrombin-stimulated platelets in the absence of fibrinogen (data not shown). In other experiments, neither BSA nor purified human fibronectin produced an increase in plt-vWF binding to thrombin-stimulated platelets in the absence of fibrinogen (Fig 1B; broken lines). In the studies that measured plt-vWF binding to platelets, inhibition of plt-vWF binding was noted at high concentrations of fibrinogen (Fig 1A) and fibronectin (Fig 1B). The additional finding that fibrin(ogen) in the presence of the GPRP tetrapeptide has no effect on the binding of released plt-vWF to thrombin-stimulated platelets supports our impression that the fibrin must bind to the platelet surface to have any effect on plt-vWF binding. These results are in agreement with our earlier work that demonstrated that in the absence of fibrin(ogen) released plt-vWF binds to the platelet surface GPIIb/IIIa.[6]

The decreased plt-vWF binding noted in the present experiments is a consequence of competition of plt-vWF with fibronectin or fibrin for this GPIIb/IIIa site. In both our present and prior studies, plt-vWF binding to thrombin-stimulated platelets was never completely inhibited by either fibrinogen or fibronectin. This suggests the possibility of multiple binding sites for plt-vWF on the platelet surface.

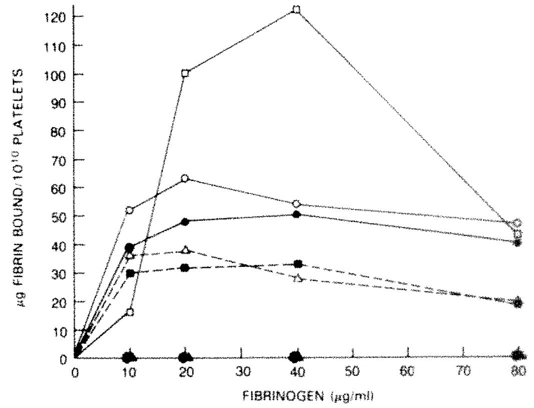

Fig 2. Binding of radiolabeled human fibrin(ogen) to activated platelets. Aliquots of platelets (200 μL at 200,000/μL) were incubated with increasing concentrations of ^{125}I-radiolabeled, DEAE-purified normal human fibrinogen and stimulated by thrombin (○) or collagen (⬤) or by thrombin in the presence of 1 μmol/L fibrinogen fragment D (□), 20 μg/mL 6D1 (●), 0.70 mmol/L GPRP (▲), 1 μg/mL plasma vWF, and 0.6 mg/mL ristocetin (△) or 1 μg/mL plasma vWf and 0.6 mg/mL ristocetin with 1 μmol/L fibrinogen fragment D (■). Platelets were preincubated with 20 μg/mL antibody 10E5 before incubation with fibrinogen. Results represent the means of three determinations; SEM was typically less than 15% for each point and usually less than 10%. Nonspecific binding of the radiolabeled fibrinogen in these experiments was typically 40% to 50%.

Because the enhanced binding of plt-vWF was only seen with thrombin-stimulated platelets and not with collagen-stimulated platelets, we tested whether the fibrinogen must be converted to fibrin to enhance plt-vWF binding. Fibrin(ogen) binding to non-GPIIb/IIIa sites on the platelet was assessed by using platelets preincubated with saturating amounts of anti-GPIIb/IIIa monoclonal antibody (10E5; 20 μg/mL). In these studies, collagen-stimulated platelets bound no radiolabeled fibrinogen, whereas significant amounts of fibrin(ogen) were bound to thrombin-stimulated platelets (Fig 2). The addition of monoclonal antibody 6D1 to platelets already preincubated with monoclonal antibody 10E5 resulted in approximately a 25% reduction in fibrin(ogen) binding to thrombin-stimulated platelets as compared with thrombin-stimulated platelets preincubated with 10E5 alone. The presence of purified plasma vWF and ristocetin (1 μg/mL and 0.6 mg/mL, final concentrations, respectively) resulted in a 30% to 60% reduction of fibrinogen binding with or without added fibrinogen fragment D (1 μmol/L) (Fig 2). In contrast, the addition of fibrinogen fragment D to prevent fibrin polymerization caused a 59% and 126% increase in fibrin binding (compared with thrombin-stimulated platelets with 10E5 alone) at fibrinogen concentrations of 20 and 40 μg/mL, respectively (Fig 2).

The amount of fibrinogen cleaved to fibrin monomer after thrombin treatment was measured as free FP-A. Incubation of fibrinogen with collagen did not result in FP-A production, and the GPRP tetrapeptide did not interfere with the release of FP-A by thrombin (data not shown). The purified fibrinogen did not contain free FP-A. Because fibrin monomer spontaneously polymerizes to form fibrin polymer, we assessed this reaction by measuring light absorbance at 350 nm; this measurement allowed us to calculate the relative amounts of fibrin monomer and polymer present. These data are presented in Table 1. When enhanced binding of plt-vWF and fibrin monomer occurs (as in Figs 1 and 2), Table 1 indicates that fibrin monomer is present in a relative excess over fibrin polymer. These data suggest that fibrin monomer or small fibrin polymers both bind to platelets (at non-GPIIb/IIIa sites) and induce the binding of released plt-vWF to GPIb.

To investigate the relationship between plt-vWF and fibrin monomer binding to the platelet surface, we analyzed the amount of platelet-bound fibrin monomer (Fig 2) *v* the increase in platelet-bound plt-vWF (Fig 1). These data are presented in Fig 3. As indicated, the threshold quantity of

Table 1. Fibrin Monomer/Polymer Formation in the Presence of Bovine Fibrinogen Fragment D

Fibrinogen (µg/mL)	Fibrinogen (% Cleaved)†	Maximum‡ Fibrin (µg/mL)	Percent Polymer Formation§	Total Polymer‖	Monomer¶ (µg/mL)	MMonomer/ Polymer
10	98	9.8	11.8	1.16	8.84	7.6
20	81	16.2	22.1	3.58	12.62	3.5
40	70	28.0	28.2	7.90	20.1	2.5
80	40	32.0	65.0	20.8	11.2	0.5

Fibrinogen was incubated with human α-thrombin (0.5 U/mL, final concentration) in the presence of bovine fibrinogen fragment D (1 µmol/L).

†Percent fibrinogen cleavage is calculated by the following formula: FP-A measured × 100/FP-A predicted.

‡The maximum concentration of fibrin is considered to be equal to the concentration of fibrinogen × percent fibrinogen cleavage/100.

§The percent fibrin polymer formation is determined spectrophotometrically by measuring light absorbance at 350 nm of a suspension of thrombin-cleaved fibrinogen (see Materials and Methods).

‖Total fibrin polymer is calculated by the formula: maximum fibrin polymer × percent polymer/100.

¶Concentration of fibrin monomer is calculated by the following formula: [maximum fibrin − fibrinogen] × (percent cleavage/100) × percent polymer/100.

platelet-bound fibrin monomer required to mediate enhanced plt-vWF binding to platelets is 45 µg/10^{10} platelets. Plt-vWF binding increases only when bound fibrin monomer exceeds this value.

DISCUSSION

These experiments indicate that fibrin monomer mediates plt-vWF binding to the glycocalicin portion of platelet GPIb. The initial observation showed that fibrin, when present in small concentrations, resulted in increased surface expression of plt-vWF on thrombin-stimulated platelets. Confirmation that this effect was specific for fibrin was demonstrated by the inability of other purified proteins (eg, bovine serum albumin and human fibronectin) to enhance plt-vWF binding (Fig 1B). The ability of fibronectin to interact with platelets was confirmed by the inhibition of plt-vWF binding to platelets when fibronectin is present at high concentrations (Fig 1B). Studies measuring the generation of FP-A or using collagen as the platelet agonist confirmed that fibrin and not fibrinogen was responsible for the enhanced plt-vWF binding to platelets.

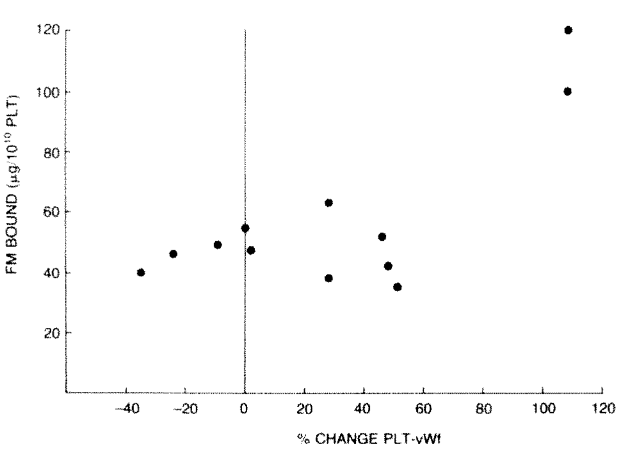

Fig 3. Graph of bound fibrin monomer v percent change in plt-vWF binding. Each point represents the mean of three points. The best fit for this curve is a three-parameter hyperbolic function with the equation $1/Y = A + Bx$ where $Y = FM$ (fibrin monomer) bound, x = percent change in plt-vWF, $A = 0.0207 \pm 0.0015$, and $B = 0.000109 \pm 0.000015$. The y-intercept by this equation is 44.9 ± 6.3. P value for curve fit < .001 by the F test.

Studies with the GPRP tetrapeptide, which prevents both fibrin polymerization[18] and fibrin/fibrinogen binding to platelet GPIIb/IIIa,[11,19] demonstrated that binding of the fibrin to the platelet is necessary to induce plt-vWF binding (Figs 1, 2, and 3). Fibrin polymerization is inhibited by either the GPRP tetrapeptide or the fragment D isolated from fibrinogen.[20] However, the binding of the fibrin monomer to the platelet surface is prevented by the GPRP tetrapeptide, whereas fragment D does not interfere with fibrin binding to platelets (Fig 2). The importance of this difference is critical. These experiments demonstrate that fibrin monomer must bind to the platelet surface to have any effect on plt-vWF binding, ie, fragment D enhances plt-vWF binding whereas GPRP has no effect (Fig 1).

The studies using a monoclonal antibody directed against GPIb and a monospecific anti-glycocalicin (α-GC) antiserum suggest that the glycocalicin portion of GPIb serves as the receptor for fibrin monomer–induced plt-vWF binding. Further evidence that GPIb serves as the receptor site for fibrin monomer–induced plt-vWF binding are the results of the studies with Bernard-Soulier platelets in which fibrin monomer does not increase plt-vWF binding while fibrin(ogen) inhibits plt-vWF binding. The degree of inhibition is quite similar to that observed with normal platelets incubated with the α-GC antiserum. These data are consistent with our earlier studies demonstrating binding of released plt-vWF to platelet GPIIb/IIIa[6] and demonstrate the inhibition of this binding in the presence of polymerized fibrin. These data along with the results obtained when fibrin polymerization is prevented by fibrinogen fragment D suggest that fibrin polymer is the form of fibrin that binds to GPIIb/IIIa and that fibrin monomer (or very small fibrin oligomers) binds to GPIb.

In experiments designed to test whether GPIb serves as a receptor for fibrin monomer, we used platelets in which the vWF binding site on GPIb was occupied by monoclonal antibody 6D1 or by plasma vWF. In these studies, fibrin(ogen) binding to GPIIb/IIIa is completely blocked by monoclonal antibody 10E5. The finding of a significant reduction in the binding of fibrin to these platelets indicates that a site on the glycocalicin portion of GPIb is involved in the binding of fibrin monomer to platelets. Furthermore, by the use of platelets in which plasma vWF is bound to GPIb in the presence of ristocetin we were able to determine that surface-bound vWF does not serve as a receptor for fibrin or fibrin monomer. This suggests that a vWF–fibrin monomer complex does not form on the platelet surface, and if such a complex formation is an important step in the binding of plt-vWF to the platelet GPIb, the complex must form before the vWF binds to the platelet. Because fibrin binding is not completely inhibited in these experiments, our data suggest that either the site on glycocalicin to which fibrin monomer binds is not identical to that for vWF or that there are additional non-GPIb, nonGPIIb/IIIa binding sites for fibrin monomer on the platelet surface.

Our results indicating enhanced binding of fibrin monomer to platelets are consistent with earlier studies by Niewiarowski et al[21,22] who demonstrated binding of polymerizing fibrin but not native fibrinogen or fully polymerized fibrin to washed platelets. The recent report of Loscalzo and et al[12] that documents binding of plasma vWF and purified glycocalicin to immobilized fibrin monomer shows that plasma vWF enhances platelet incorporation into polymerizing fibrin. They suggest that a vWF–fibrin monomer complex is formed that subsequently binds to platelet glycocalicin. These studies in concert with our observations suggest that the mechanism of enhanced plt-vWF binding in the presence of low concentrations of fibrinogen involves the binding of fibrin monomer to the glycocalicin portion of platelet GPIb. Our studies showing inhibition of plt-vWF binding in the presence of the GPRP tetrapeptide suggest that fibrin monomer binding to platelets is required to induce plt-vWF binding to platelets. We have shown that an epitope on glycocalicin serves as the receptor for this fibrin monomer–dependent binding of plt-vWF.

The clinical significance of this phenomenon is speculative at this time, although the recent finding of Michelson et al[23] localizing the ristocetin-dependent plasma vWF binding site on GPIb to glycocalicin suggests that fibrin monomer–induced binding of plt-vWF to glycocalicin may have a function similar to that described for the binding of plasma vWF to platelets in the presence of ristocetin. Under conditions that may favor fibrin monomer formation (eg, disseminated intravascular coagulation, the environment within an evolving thrombus) platelet adhesion may be maximized by this process of fibrin monomer–induced plt-vWF binding. Inasmuch as we do not find increased fibrin monomer binding to platelets with plasma vWF prebound to GPI (Fig 2), we believe that vWF bound to GPIb is not the receptor for fibrin monomer binding. The platelet receptor for fibrin monomer is not determined in this study. However, from our data, we propose the following model: fibrin monomer is formed and binds initially to the platelet, possibly at a site near the vWF binding site on GPIb. This interaction results in a conformational change in the glycocalicin portion of GPIb, which allows the binding of vWF to its receptor on GPIb. An alternative possibility involves the binding of a fibrin monomer–vWF complex to the platelet GPIb through the binding of the fibrin monomer component of the complex to the platelet.

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Interleukin-2 Production and Response to Interleukin-2 by Peripheral Blood Mononuclear Cells From Patients After Bone Marrow Transplantation: II. Patients Receiving Soybean Lectin–Separated and T Cell–Depleted Bone Marrow

By Karl Welte, Carolyn A. Keever, Janet Levick, Mary Ann Bonilla, Vincent J. Merluzzi, Roland Mertelsmann, Robert Evans, and Richard J. O'Reilly

The ability of peripheral blood mononuclear cells (PBMC) to produce and respond to interleukin-2 (IL-2) was evaluated in 50 recipients of HLA-identical bone marrow (BM) depleted of mature T cells by soybean agglutination and E rosetting (SBA$^-$E$^-$BM). In contrast to our previous findings in recipients of unfractionated marrow, during weeks 3 to 7 post-SBA$^-$E$^-$BM transplantation (BMT), PBMC from the majority of patients spontaneously released IL-2 into the culture medium. This IL-2 was not produced by Leu-11$^+$ natural killer cells, which were found to be predominant in the circulation at this time, but by T11$^+$, T3$^+$, Ia antigen-bearing T cells. The IL-2 production could be enhanced by coculture with host PBMC frozen before transplant but not by stimulation with mitogenic amounts of OKT3 antibody, thus suggesting an in vivo activation of donor T cells or their precursors by host tissue. Spontaneous IL-2 production was inversely proportional to the number of circulating peripheral blood lymphocytes and ceased after 7 to 8 weeks post-SBA$^-$E$^-$BMT in most of the patients. In patients whose cells had ceased to produce IL-2 spontaneously or never produced this cytokine, neither coculture with host cells nor stimulation with OKT3 antibody thereafter induced IL-2 release through the first year posttransplant. Proliferative responses to exogenous IL-2 after stimulation with OKT3 antibody remained abnormal for up to 6 months post-SBA$^-$E$^-$BMT, unlike the responses of PBMC from recipients of conventional BM, which responded normally by 1 month post-BMT. However, the upregulation of IL-2 receptor expression by exogenous IL-2 was found to be comparable to normal controls when tested as early as 3 weeks post-SBA$^-$E$^-$BMT. Therefore, the immunologic recovery of proliferative responses to IL-2 and the appearance of cells regulating in vivo activation of T cells appear to be more delayed in patients receiving T cell–depleted BMT. Similar to patients receiving conventional BMT, however, the ability to produce IL-2 after mitogenic stimulation remains depressed for up to 1 year after transplantation.

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IT HAS BEEN WELL DOCUMENTED that patients after bone marrow transplantation (BMT) demonstrate severe deficiences of cellular and humoral immunity.[1-6] The inability to produce normal amounts of interleukin-2 (IL-2) is considered to be one possible mechanism for deficient immune function post-BMT.[7-12]

IL-2 is one of the major cytokines responsible for clonal expansion and function of T cells,[13-15] natural killer (NK) cells,[16-18] and lymphokine activated killer cells in vitro and in vivo.[19,20] Defective mitogen-induced IL-2 production has been documented in several immunodeficiency states including severe combined immunodeficiency, acquired immunodeficiency syndrome, and common variable immunodeficiency.[14] We previously reported[10] that when using OKT3 antibody as a T cell–specifc mitogen[21-23] peripheral blood mononuclear cells (PBMC) from patients receiving allogeneic, HLA-identical unfractionated bone marrow (BM) were deficient in their production of IL-2 up to 18 months post-BMT. This defective IL-2 production was associated with severely depressed proliferative responses to OKT3. However, in the presence of exogenous IL-2, PBMC from the majority of patients showed normal proliferation.[10]

Relatively little is known about the immune function of PBMC from patients with leukemia after receiving T cell–depleted BMT. In our center, a technique was developed to remove from BM mature blood cells including T lymphocytes by soybean agglutination and subsequent E rosette depletion (SBA$^-$E$^-$BMT).[24,25] The enriched source of hematopoietic progenitor cells isolated by this procedure is adequate to ensure full engraftment of all cellular BM elements and recovery of marrow function.[25] Such grafts prevent acute and chronic forms of graft-*v*-host disease (GvHD) posttransplant in both HLA-identical and HLA-nonidentical recipients.[25,26] The kinetics of hematopoietic recovery do not differ significantly from that in patients receiving unfractionated BM. However, as shown in this study, the pattern of reconstitution of T cell subpopulations and T cell functions in the first 3 months after BMT is different from those in patients receiving unfractionated BMT.[10]

We have sequentially examined PBMC from patients receiving T cell–depleted BMT for (*a*) their ability to produce IL-2, (*b*) their IL-2 receptor expression, and (*c*) their ability to proliferate in response to mitogen with and without the addition of IL-2.

MATERIALS AND METHODS

Patients and controls. The study group consisted of 50 recipients of SBA$^-$E$^-$, allogeneic, HLA-identical BM: 13 patients with acute lymphoblastic leukemia in first, second, or third remission; two patients with non-Hodgkin's lymphoma; 13 patients with acute myeloid leukemia in first or second remission; 19 patients with

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chronic myelogenous leukemia; and three patients with myelodysplastic syndrome. Pretransplant conditioning was with cyclophosphamide (60 mg/kg/d for two days) and hyperfractionated total body irradiation (1,320 rad or 1,440 rad). No drug prophylaxis was administered posttransplant to prevent GvHD. All patients tested in this series achieved durable engraftment confirmed by cytogenetics and full hematopoietic reconstitution.

Forty-three patients were tested at least once during weeks 3 to 7 post-BMT, and 26 of the 43 patients were tested more than once (two to five times) during this time period. Of the 43 patients, 35 patients subsequently were tested one or more times during weeks 8 to 14 post-BMT. Seven additional patients were tested for the first time during this time period. Eighteen patients in the study were repeatedly tested up to 1 year post-BMT. Normal controls consisted of 51 laboratory personnel aged 20 to 45 years.

The protocol for this study was subject to initial approval and subsequent annual review by the human subject review committee of Memorial Sloan Kettering Cancer Center. All participants in the study signed committee approved consent forms.

Monoclonal antibodies. OKT3, OKT4, OKT8, and OKT11 monoclonal antibodies (MoAbs) were purchased from Ortho Pharmaceuticals (Raritan, NJ). Leu-11b antibody was purchased from Becton Dickinson, (Mountain View, CA). Leu-2, Leu-3, Leu-4, and Leu-5 antibodies were generated as culture supernatants in our laboratory by one of us (Dr R. Evans). The hybrid line secreting Leu-7 was obtained from the American Tissue Culture Collection (Rockville, MD). Anti-Tac MoAb (ascites fluid) was kindly provided by Dr T. Waldmann, National Institutes of Health, Bethesda, MD; NKH1 MoAb by Drs R. Schmidt and J. Ritz, Dana Farber Cancer Center, Boston; and PL8 (anti-HLA class II) by Dr K. Horibe, Memorial Sloan-Kettering Cancer Center, NY.

Cell preparations. PBMC from patients or healthy human volunteers were separated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and were kept in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (FCS) during all subsequent incubation and washing procedures. Adherent cells were removed by incubation on Falcon tissue culture dishes (no. 3003, Falcon Labware, Oxnard, CA) as described[23] and are referred to as peripheral blood lymphocytes (PBL). In some experiments E^+ cells were separated from PBMC by rosetting with neuraminidase-treated sheep erythrocytes as described.[27]

Analysis of T cell surface antigens. Immunofluorescence analyses were performed on a FACS IV (Becton Dickinson). PBMC (10^6 cells) were incubated with appropriate dilutions of MoAb (1:4 for culture supernatants, 1:10,000 for anti-Tac, 1:40 for all other antibodies) for 40 minutes at 4°C, washed twice, and then incubated with a 1:40 dilution of affinity-purified goat antimouse IgG, $F(Ab')_2$-fluorescein isothiocyanate (FITC) (Cappel Laboratories, Cochranville, PA) in the same way. After washing twice, the cells were suspended in 1 mL phosphate-buffered saline for analysis. All incubations and centrifugation steps were done at 4°C. Background fluorescence was determined by using a supernatant containing an IgG1κ protein of diluted normal mouse serum instead of the first antibody.

Proliferation assay. The separated PBMC (1×10^6 cells/mL) or E^+ cells (supplemented with 10% macrophages, 1×10^6 cells/mL) were incubated in triplicate cultures (no. 3596 culture plates, Costar, Inc, Cambridge, MA) in the presence or absence of OKT3 antibody, in the presence and absence of phorbol-myristate-acetate (PMA), and in the presence or absence of exogenous IL-2 in a total volume of 200 μL. The concentrations used were OKT3, 2.5 ng/mL; PMA, 2.5 ng/mL; and IL-2, 20 U/mL. After 72 hours, the cultures were pulsed for four hours with ^3H-thymidine (0.5 μCi/well, New England Nuclear, Boston) and proliferation measured as described previously.[10,23]

Assay for IL-2 production and IL-2 activity. Identical cultures (see Proliferation Assay, in the absence of exogenous IL-2) were incubated for 24 hours, and 100 μL of supernatant was removed to be assayed for IL-2 activity. IL-2 was measured by using the assay previously described by Gillis et al.[28] Units of IL-2 activity were determined by using an IL-2 standard provided by the National Institutes of Health Biological Response Modifers Program.

BM separation and IL-2 production by $SBA^-E^-BM^-$. Donor BM cells were enriched for progenitor cells and depleted of mature blood cells including T lymphocytes by using soybean agglutinin and E rosetting by a method originally developed by Reisner et al.[24,25] The SBA^-E^-BM cells were incubated at 10^6 cells/mL in RPMI 1640 supplemented with 10% FCS in the absence and presence of OKT3 antibody (2.5 ng/mL). After incubation at 37°C for 24 hours, supernatants of the cells were collected and tested for IL-2 activity as described.[28,29]

IL-2 source. For this study we used human recombinant IL-2, kindly provided by CETUS, Emeryville, CA.[30] In some of the studies highly purified IL-2 (hpIL-2) was used in parallel, which was purified from lymphocyte-conditioned medium (Lymphocult, Biotest, Fairfield, NJ) as described.[29] Recombinant IL-2 and hpIL-2 gave qualitatively and quantitatively identical results in these experiments. Therefore, results are detailed for recombinant IL-2 only.

Antibody plus complement depletions. PBL were treated with MoAb plus complement (C') to remove selected populations of cells. All of the MoAbs used were free of sodium azide. The cells were resuspended in 0.1 mL/10^6 cells of antibody or medium alone. The optimum dilution established for OKT3, OKT11, OKT4, and OKT8 was 1:20; Leu-11b, 1:50, NKH-1, 1:125; and PL8, 1:2. After a 60-minute incubation at 4°C the cells were centrifuged, and the MoAb was decanted. A volume of 0.5 mL of a pretested dilution (1:5) of low-toxicity rabbit C' (Pel-Freez, Rogers, AR) was added, and incubation was continued at 22°C for 60 minutes. The cells were washed twice with RPMI-10% FCS, counted, and adjusted to the same volume as the C' control (cells incubated with C' but without MoAb) before culturing. PBL that had been incubated overnight at 37°C to shed bound antibodies were restrained with directly FITC conjugated antibodies to monitor T cell depletion. Less than 5% of the relevant population could be detected. PBL that had been depleted of Leu-11^+ or NKH-1^+ cells had no lytic activity to K562 in four-hour, ^{51}Cr release assays.

Mixed cell culture. Mixed cell cultures were performed in RPMI 1640 plus 10% FCS. Responder cells (10^5 cells) were cocultured with irradiated, previously frozen pretransplant PBMC (10^5 cells) from the patients or their donors at 37°C and 5% CO_2 for 72 hours. The supernatant was removed and tested for the presence of IL-2. Controls included responders incubated without stimulators and irradiated stimulators incubated alone. In one experiment, mixed cell cultures between irradiated donor SBA^-E^-BM cells (obtained on the day of transplant) and pretransplant patient PBMC and donor PBMC were performed.

Statistical analysis. Statistical evaluation was performed by using the nonparametric two-sample rank test (Mann-Whitney test).

RESULTS

PBMC from patients 3 to 7 weeks post-BMT were cocultured with medium alone, PMA, OKT3, and a combination of T3 and PMA, and the amount of IL-2 secreted into the medium was measured. The results in Fig 1 show that unlike

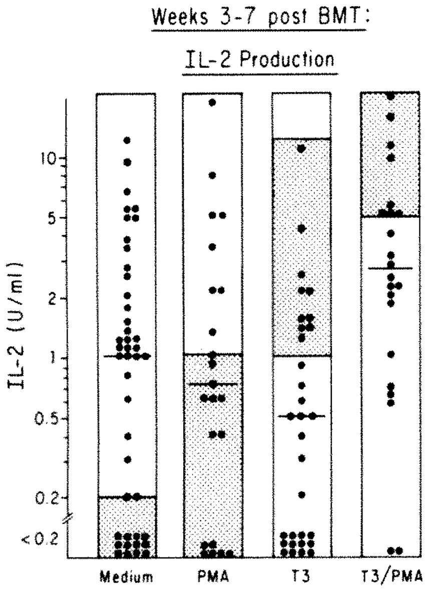

Fig 1. IL-2 production by PBMC from patients 3 to 7 weeks post-BMT. PBMC from patients were cultured (10^6 cells/mL) in RPMI 1640 and 10% FCS in the absence (medium) or presence of stimulators: PMA, 2.5 ng/mL; T3 (OKT3), 2.5 ng/mL; or PMA plus T3. After 24 hours the supernatants were tested for IL-2 activities. The results are shown for each patient only once at the time of maximal IL-2 release without stimulator (medium). The shaded area represents the 100% range of normal donors (n = 51). ———, median.

Table 1. IL-2 Production by Posttransplant PBMC After Coculture With Pretransplant Host or Donor PBMC

Patient No.	Week Post-BMT	Day 1*	Day 3†	Stimulators (Cells/Well) Pretransplant Host PBMC 10^5	5×10^4	Donor PBMC (10^5)
526	6	6.5‡	6.6	9.4	7.4	11
	7	3.5	3.1	2.1	2.3	3.6
536	3	0.6	1.0	1.3	1.9	0
	8	0	1.9	1.3	1.7	0
514	3	0.9	0	3.5	1.8	NT
	4	NT	0	2.8	1.7	NT
539	3	1.2	0	2.0	1.7	1.0
483	3	0	0.6	NT	1.5	NT
527	5	4.0	0	NT	1.7	NT
600	5	2.0	1.4	2.0	1.4	NT

Abbreviation: NT, not tested.

*Spontaneous IL-2 production after 24 hours of culture.

†PBMC obtained from patients at the indicated times post-BMT were cultured for 72 hours in medium (RPMI 1640 plus 10% FCS) or cocultured with host or donor PBMC cryopreserved pretransplantation. Secreted IL-2 was measured as described in Materials and Methods. Results are expressed as units of IL-2/mL supernatant.

‡Data are units of IL-2/mL.

normal controls PBMC from 31 of 43 patients spontaneously secreted detectable amounts of IL-2 (0.2 to 12 U/mL; median, 1.0 U/mL) into the medium ($P < .001$). The addition of PMA (2.5 ng/mL) or OKT3 antibody (2.5 ng/mL) to the medium did not increase IL-2 production. OKT3 plus PMA, however, increased production, but the levels achieved were below the normal range of the control group ($P < .01$), which is shown as the shaded area (100% range). The IL-2 produced by PBMC from patients after OKT3 stimulation was no greater than the spontaneous IL-2 production. All eight of the patients whose PBMC produced normal amounts of IL-2 after stimulation with OKT3 plus PMA were also spontaneous IL-2 producers (>1 U/mL).

Table 1 shows results from cocultures between weeks 3 to 7 posttranspant PBMC and irradiated pretransplant host PBMC and donor PBMC, respectively. Spontaneous IL-2 production was measured on days 1 and 3 of culture, and stimulated IL-2 production was measured on day 3. Compared with day 1, spontaneous IL-2 production on day 3 of culture was unchanged in two experiments, increased in three experiments, and decreased in four experiments. In eight of ten experiments, irradiated pretransplant host PBMC stimulated higher production of IL-2 than that seen in medium alone (day 3). In three of five experiments donor PBMC also increased the production of IL-2 compared with the medium alone, but in only one patient (no. 526) was this production greater (11 U/mL) than that stimulated by coculture with pretransplant host cells (9.4 U/mL). In contrast, when the donor's own PBMC were cultured alone or with irradiated prestransplant host cells, no detectable IL-2 was produced. Furthermore, irradiated host or donor stimulator cells incubated alone (data not shown) did not generate IL-2.

Cultures of SBA$^-$E$^-$BM specimens from 40 donors in medium alone or in the presence of OKT3 (2.5 ng/mL) did not result in the release of detectable amounts of IL-2. In contrast, unfractionated BM (n = 16) under the same conditions released IL-2 (1 to 4 U/mL) when stimulated with OKT3, which indicated the presence of T cells in the unfractionated marrow sample.

Mixed cell cultures using irradiated donor SBA$^-$E$^-$BM cells as stimulators and host pretransplant PBMC or PBMC as responders also did not result in IL-2 production, which indicated that the donor SBA$^-$E$^-$ marrow does not contain cell populations capable of stimulating IL-2 production by either the donor or the HLA-identical host. As expected, however, the SBA$^-$E$^-$BM fraction was able to induce IL-2 production by a third-party control PBMC (data not shown).

Phenotype of the T cells during weeks 3 to 7 post-BMT. Phenotyping of PBMC from patients was performed at the same post-BMT time as IL-2 production was measured. Most striking was the consistently high percentage of cells with the NK-associated antigens (Leu-11 and Leu-7). Leu-5$^+$ (CD2) cells were consistently in significant excess of Leu-4$^+$ (CD3) cells, which suggested the presence of cells with the Leu-4$^-$, Leu-5$^+$, Leu-11$^+$ or NK cell phenotype as reported by others.[6] It is also possible, however, that the discrepancy between Leu-4$^+$ and Leu-5$^+$ cells reflects the presence of immature prethymic Leu-5$^+$, Leu-4$^-$, Leu-3$^-$, Leu-2$^-$ cells (Table 2).

The phenotype of PBMC from patients who did or did not spontaneously generate IL-2 was not different with the

Table 2. Surface Marker Analysis of PBMC From Patients 3 to 7 Weeks Post-BMT

		Antibodies (Percentages of Positive Cells)						
Patient No.	IL-2 (U/mL)	Leu-5 (CD2) [83 ± 5]	Leu-4 (CD3) [75 ± 7]	Leu-3 (CD4) [45 ± 10]	Leu-2 (CD8) [28 ± 8]	Leu-7 [20 ± 7]	Leu-11 [15 ± 6]	Tac [2 ± 2]
Spontaneous IL-2 producer								
488	13.2	61	12	11	27	34	49	13
472	5.5	49	8	3	47	34	NT	NT
514	5.2	82	56	22	52	57	28	8
448	5.0	25	17	19	21	6	NT	18
464	4.5	NT	26	NT	21	26	NT	9
494	3.5	50	51	14	46	NT	21	9
489	1.5	41	30	4	25	40	59	NT
417	1.5	66	49	16	43	95	NT	17
415	1.5	NT	26	9	18	12	NT	NT
404	1.3	NT	31	11	39	37	NT	NT
471	1.3	34	11	5	28	41	NT	11
438	1.2	NT	71	17	55	NT	NT	NT
491	1.1	57	NT	7	32	34	77	5
483	1.1	59	16	9	28	50	29	6
493	1.0	62	19	15	25	16	22	4
Mean ± SD		53 ± 16	30 ± 19	12 ± 6	34 ± 12	37 ± 23	41 ± 21	10 ± 5
Nonproducers								
419	0	86	84	5	80	35	NT	NT
467	0	NT	28	21	32	26	NT	NT
469	0	84	39	31	37	10	NT	0
475	0	24	3	12	13	13	19	NT
479	0	46	17	8	9	32	2	0
482	0	24	15	10	16	NT	14	3
487	0	51	17	8	32	19	43	4
513	0	87	40	7	46	73	60	7
Mean ± SD		57 ± 28	30 ± 25	13 ± 9	33 ± 23	30 ± 21	28 ± 23	3 ± 3

Control values are in brackets.

exception of an increased level of Tac^+ cells in the spontaneous IL-2–producing patients (10% ± 5% Tac^+ cells). This difference compared with normal controls was significant ($P < .01$).

Phenotype of the IL-2–producing cells. MoAb plus C′ treatment of PBL from spontaneous IL-2 producers showed reduced IL-2 production when T11, T3, and Ia plus C′ were used. The results from five patients tested at different times post-BMT are shown in Table 3. Surprisingly, Leu-7, Leu-11b, or NKH1 plus C′ treatment of PBL did not reduce the IL-2 production in any experiment, which suggested that although this population of cells predominates at this time the cells producing IL-2 were not NK cells. T4 or T8 plus C′ treatment of PBL had only minor effects on the IL-2 production.

IL-2 is able to induce its own receptor on PBMC from patients after BMT. In five patients (3 to 7 weeks post-BMT) we studied the ability of IL-2 to upregulate its own receptor expression on PBMC as has been shown for normal PBMC[31,32] (Table 4). The percentage of Tac+ PBMC before culture was between 7% and 9%. Culturing the PBMC from these five patients without mitogenic stimulation but in the presence of IL-2 (100 U/mL) led to an increase of the

Table 3. Phenotype of IL-2 Producer Cells: Reduction of IL-2 Production by Treatment of PBMC With Antibody Plus C′

Antibody	IL-2 (Percentage of C′ Control*)	No. of Experiments (No. of Patients)
Complement control	100	12 (5)
T11 + C′	35	10 (5)
T3 + C′	28	4 (4)
T4 + C′	60	3 (3)
T8 + C′	93	3 (3)
Leu-7 + C′	115	1 (1)
Leu-11 + C′	101	6 (4)
NKH1 + C′	120	2 (2)
Ia + C′	23	2 (2)

*Mean C′ of control, 3 U/mL.

Table 4. Increase of Tac Antigen Expression of PBMC Cultured in Medium Containing IL-2

	Percentage of Tac+ Cells	
Patient No.	Day 0	Days 3-4
1	7	20
2	8	33
3	8	27
4	8	26
5	9	17
Median (range)	8 (7-9)	26 (17-33)

PBMC obtained from patient 3 to 7 weeks post-BMT were cultured for three to four days in medium (RPMI 1640 plus 10% FCS) in the presence of recombinant IL-2 (100 U/mL). Tac antigen expression was measured as described in Material and Methods. A healthy control tested at the same time under the same conditions had 0% Tac+ cells at day 0 and 21% Tac+ cells at day 4.

percentage of Tac+ cells from 8% (7% to 9%) to 26% (17% to 35%) at days 3 to 4 in culture. PBMC from a simultaneously tested normal control increased from 0% to 21% Tac+ cells at day 4.

The kinetics of spontaneous IL-2 production. As shown in Table 5, the maximal spontaneous IL-2 production occurred during the first 7 weeks post-BMT. After 8 weeks and at later times post-BMT, the majority of patients no longer spontaneously produced IL-2, and the pattern of mitogen-induced IL-2 production became more similar to that of patients receiving unfractionated BMT.[10] The amount of spontaneous IL-2 produced by any one patient during the first 7 weeks post-BMT varied considerately (Fig 1). We were interested to determine the time course and factors that may affect production by PBMC from the patients under study. In Fig 2, we have detailed the course of IL-2 production along with WBC and lymphocyte recovery of six patients who represent different patterns of spontaneous IL-2 production. Patient A (Fig 2A) spontaneously produced IL-2 from weeks 3 to 6 post-BMT, whereas patient E (Fig 2E) never produced detectable amounts of IL-2 during this period. Patients B, C, D, and F (Fig 2B, 2C, 2D, 2F) produced IL-2 only for a short period of time (up to week 5 post-BMT). As can be seen, the spontaneous IL-2 production was inversely correlated with the recovery of the number of WBC and absolute lymphocyte count (ALC): patients E and F, who experienced a rapid recovery of WBC, either did not produce IL-2 spontaneously (2E) or produced it only very early post-BMT (3 weeks; Fig 2F). In contrast, patients A and B, who experienced a slower return of WBC, had a high level of spontaneous IL-2 production for a protracted period post-BMT.

The inverse correlation between ALC and the amount of IL-2 produced in vitro by PBMC was examined for all tests (n = 85) performed on patients (n = 43) between weeks 3 and 7 post-BMT. High spontaneous IL-2 producers (>3 U/mL) were found only in the group with ALC <500/μL. The difference in spontaneous IL-2 production between patients with ALC <500/μL was statistically significant ($P < .001$).

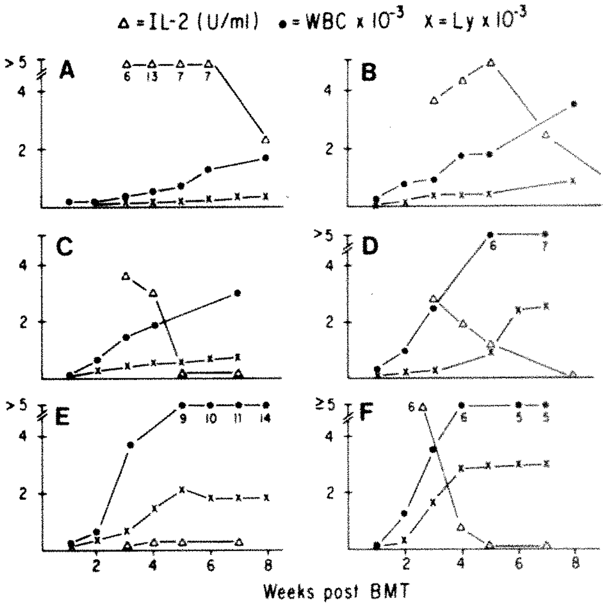

Fig 2. Time course of spontaneous IL-2 production (△), WBC recovery (●), and absolute lymphocyte number (x) in six representative patients tested more than three times during the first 7 weeks post-BMT. The numbers below the symbols point out the exact values when higher than or equal to 5.

Patients A, C, and E were also tested for the percentage of Tac+ cells; patient A was 13% at week 4, 11% at week 5, 5% at week 7, and 2% at week 8. In patient C the percentage of Tac+ cells was 9% at week 4 and 4% at both weeks 5 and 6. In patient E 2% of PBMC were Tac+ at week 4, 3% at week 5, and 0% at week 8. These data suggest a good correlation between the development and decline of spontaneous IL-2–producing cells and IL-2 receptor–positive cells in those patients tested.

Proliferative response to IL-2. Spontaneous prolifera-

Table 5. IL-2 Production and Proliferation of PBMC From Patients Post-BMT

	Weeks Post-BMT				
	3-7	8-12	13-26	27-52	Controls
No. experiments	80	38	20	10	64
No. tested	43	30	18	8	51
IL-2 production, day 1 of culture, U/mL; median (100% range)					
Medium	1.0 (0-12)	0 (0-2.5)	0 (0-1)	0 (0-1)	0
PMA	0.7 (0-20)	0 (0-3)	0 (0-0.8)	0 (0-0.7)	0.2 (0-1)
OKT3	0.5 (0-11)	0 (0-11)	0 (0-2)	0 (0-2.4)	3.9 (1.1-11)
OKT3/PMA	2.7 (0-20)	1.2 (0-21)	1.7 (0-7)	2.7 (0-20)	20 (4.8-80)
Proliferation, ^{3}H-thymidine uptake day 3 of culture, cpm × 10^{-3}; median (100% range)					
Medium	1 (0.3-10)	0.6 (0.1-6)	0.6 (0.2-1.4)	0.3 (0.1-2.5)	0.3 (0.2-1)
PMA	1 (0.2-8)	1 (0.2-16)	1.1 (0.1-10)	2.3 (0.4-21)	12 (1-17)
Medium/IL-2	3.7 (0.3-32)	5 (0.2-20)	3.3 (0.2-17)	6 (0.3-30)	4 (0.1-8)
PMA/IL-2	1 (0.3-21)	5 (1.2-34)	7.6 (0.3-22)	14 (8-56)	19 (7-60)
OKT3	7.6 (0.2-41)	5 (0.2-16)	3.3 (0.1-41)	19 (2-89)	66 (35-120)
OKT3/PMA	2.6 (1-29)	4 (0.2-27)	4.5 (0.2-30)	38 (4-94)	68 (39-134)
OKT3/IL-2	15 (1-76)	18 (2-82)	25 (4-103)	62 (42-97)	86 (37-120)
OKT3/PMA/IL-2	7.5 (1-35)	13 (1-79)	26 (1-71)	67 (35-132)	92 (60-127)

The normal control values are also shown as shaded areas (100% range) in Fig 1 for IL-2 production and in Fig 3 for proliferation.

tion of lymphocytes from 13 of 36 patients tested during weeks 3 to 7 post-BMT was significantly greater than PBMC from controls ($P < .01$) (Fig 3). All of these 13 patients were spontaneous IL-2 producers. Exogenous IL-2 increased the proliferation of PBMC from the majority of patients, which is consistent with the increased levels of IL-2 receptors. This IL-2–induced proliferation was also significantly different from the normal control group ($P < .001$). OKT3-induced proliferation, however, was low during weeks 3 to 7 post-BMT compared with controls, both in the absence and in the presence of IL-2 ($P < .001$). PMA was not able to increase the proliferation in the medium control or in the presence of OKT3 (Table 5).

A summary of IL-2 production and proliferative responses with and without IL-2 of PBMC from patients tested at intervals up to 1 year post-BMT is shown in Table 5. The pattern of OKT3-induced proliferative response during weeks 8 to 12 was similar to that seen during weeks 3 to 7. Statistical analysis comparing data from controls with patients up to 26 weeks post-BMT showed significant differences in all values tested ($P < .01$). Six to 12 months after BMT (weeks 27 to 52), the proliferative response to OKT3 antibody was still defective in the majority of patients (median, 19,000 cpm). However, the proliferative response by PBMC stimulated with OKT3 in the presence of exogenous IL-2 was within the normal range for all patients tested later than 27 weeks post-BMT.

DISCUSSION

In a previous study we found that patients conditioned with the same regimen as described here who received unfractionated HLA-identical BM demonstrated low proliferative response to mitogens and produced little or no IL-2 up to 18 months post-BMT in response to OKT3 antibody. Exogenous IL-2, however, was able to restore the mitogen responses to within the normal range of healthy controls as early as 1 month post-BMT.[10] Similar results are seen when using PBMC from recipients of HLA-identical SBA^-E^-BM when tested later than 6 months post-BMT. However, there were substantial differences during the early posttransplant period. Between 3 and 7 weeks post-BMT, PBMC from the majority of patients released IL-2 into the culture medium in the absence of added stimuli, thereby suggesting in vivo stimulation of T cells, a phenomenon not observed in recipients of unfractionated marrow. Furthermore, in contrast to conventional transplant recipients, the mitogen responses of PBMC at this time and until 6 months postgrafting could not be normalized by the addition of exogenous IL-2.

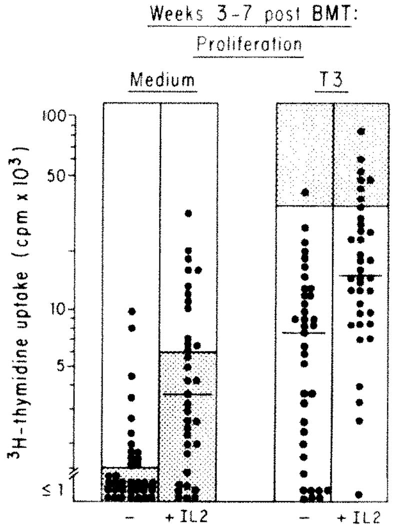

Fig 3. Spontaneous or OKT3-induced proliferation of PBMC from patients 3 to 7 weeks post-BMT in the presence or absence of exogenous IL-2 (20 U/mL). Cell proliferation was measured by ^{3}H-thymidine uptake on day 3 of culture. The shaded area represents the 100% range of normal donors (n = 51). ———, median.

The cell source of the spontaneous IL-2 production observed in recipients of T cell–depleted marrow grafts has heretofore not been defined. Brenner et al[33] recently reported that recipients of HLA-matched marrow grafts depleted of T cells with mouse MoAbs spontaneously produce IL-2, B cell differentiation factor, and γ-interferon early posttransplant. Cells in a Percoll gradient fraction enriched for large granular lymphocytes were responsible for this production, and the authors suggested that NK cells were the source of these cytokines. However, clear identification of the producer cells was not reported.

We as well as others[5,6] show a predominant population of cells with an NK phenotype developing early post-BMT. We have also found that these cells function normally in NK and lymphokine activated killer cell (LAK) assays.[34,35] However, our current studies indicate that these NK cells are not the source of spontaneously produced IL-2. C′ plus antibody lysis using Leu-11 or NKH1 antibodies did not abolish the spontaneous IL-2 release; rather, it increased IL-2 production from the remaining PBL, which suggested a possible suppressor activity of NK cells on the producer cells. Antibody plus C′ treatment of fresh PBL indicated that the IL-2–producing cell was a $T3^+$, $T11^+$, $T8^-$, Ia^+ T cell. Antibody to T4 plus C′ had a variable inhibiting effect on IL-2 production, perhaps due to a low density of the antigen on producer cells. These T cells are likely already activated because the proportion of Tac^+ T cells and spontaneous proliferation of lymphocytes from patients who spontaneously produce IL-2 was increased when compared with cells from either normal controls or patients who did not generate IL-2 spontaneously. IL-2 receptors are only expressed on T cells either activated by antigen/mitogen or stimulated by IL-2[31,32]

The stimulus provoking spontaneous IL-2 production by T cells emerging early after SBA^-E^-BMT is still unclear. In our studies, production of IL-2 by T lymphocytes emerging 3 to 7 weeks post-BMT could not be augmented by stimulation with the mitogen OKT3. The IL-2 detected in supernatants of PBMC after T3 stimulation could be accounted for by spontaneously released IL-2 because it was not increased compared with the medium control. PMA, a diacylglycerol analogue that is a potent stimulator of protein kinase C, was also not able to increase the production of IL-2. PMA in combination with the T3 antibody, however, did increase IL-2 production, which indicated that given optimal stimuli the cells were capable of producing higher, although still suboptimal, amounts of IL-2. When compared with a healthy control group ($n = 51$), the amounts of T3- and T3/PMA-induced IL-2 produced by PBMC from the patients up to 7 weeks post-BMT were well below the normal range. Our study thus indicates that T cells developing in patients early after receiving SBA$^-$E$^-$BMT are deficient in their response to mitogens as measured by lymphocyte proliferation and IL-2 production. Therefore, either the mechanism that leads to spontaneous IL-2 production has to be a very potent activator of T cells, or it activates only a subpopulation of immature T cells that do not respond to mitogens.

Given the data we have accrued to date, at least three hypotheses can be proposed to account for this spontaneous IL-2 production: (*a*) the IL-2–producing cells are residual radioresistant host T cells reactive against donor marrow whose growth and activity is normally inhibited by mature T cells in the donor marrow inoculum, (*b*) the production is by mature cells within the graft responding to minor alloantigen differences of the host, or (*c*) spontaneous IL-2 production reflects the activity of donor T cells developing within the host from early precursors in the SBA$^-$E$^-$ marrow inoculum. It is unlikely that the IL-2 production is by host cells because karyotype as well as DNA restriction length polymorphisms have indicated a clear predominance of donor-derived cells in most patients at this stage posttransplant. Furthermore, in mixed cell cultures, the generation of IL-2 at levels in excess of spontaneous production was observed with coculture of patient PBMC with irradiated host rather than donor PBMC stimulations in two thirds of the cases tested (Table 1).

Stimulation of SBA$^-$E$^-$ marrow fractions with mitogen or with host cells failed to induce IL-2 production, which rendered it unlikely that mature cells within the inoculum are the IL-2–producing cell. Most likely is the third hypothesis; indeed, persistence of spontaneous IL-2 production was inversely correlated with the temporal course of early lymphoid and hematopoietic reconstitution.

The phenotype of these IL-2–producing cells and their early development and rapid decline resembles what has been described by Lattime and Stutman in the early ontogeny of T cells in mice.[36] They described the transient presence of T cells bearing a helper phenotype that generate IL-2 in response to autologous Ia antigens present on accessory cells within the thymus. With maturation, these cells are depleted from the thymus and can be detected only as a minor population of lymphocytes in the adult spleen. Cells of a similar phenotype capable of proliferative but not cytotoxic responses to host Ia antigens have also been observed transiently in the thymus and spleens of lethally irradiated mice that have been reconstituted with T cell–depleted, H-2–incompatible marrow.[37,38]

We tested the response of PBMC from the patients post-SBA$^-$E$^-$BMT to exogenously added IL-2 in two assay systems: (*a*) spontaneous and mitogen-induced proliferation and (*b*) regulation of the IL-2 receptor expression by IL-2. Our results suggest that T cells from SBA$^-$E$^-$BMT recipients have a delayed response to OKT3 in the presence of exogenous IL-2 compared with those from patients receiving conventional BMT.[10] Although PBMC from all recipients of unfractionated BM proliferated normally to OKT3 plus IL-2 as early as 1 month post-BMT,[10] recipients of SBA$^-$E$^-$BM required as long as 6 months to regain normal proliferation under the same experimental conditions (Table 5). PMA as a costimulator did not significantly increase the T3-induced proliferation of PBMC from recipients of SBA$^-$E$^-$BMT.

The increased IL-2 receptor expression of freshly obtained patient PBMC is shown in Table 2. The ability of IL-2 to induce its own receptor on T cells from patients up to 7 weeks post-BMT was also tested. As reported for normal T cells,[31,32] IL-2 was able to upregulate the IL-2 receptor expression as judged by staining with the Tac MoAb in all patients tested. The highest percentage of Tac$^+$ cells was detected at days 2 to 3 of culture in IL-2–containing medium. This finding is of interest in that it suggests that even though the T cells are unable to proliferate normally in response to IL-2 the pathway of IL-2–stimulated IL-2 receptor induction seems to be intact. Alternatively, it is possible that NK cells, which represent a high percentage of the lymphocytes detected early after BMT, are able to respond to IL-2 with increased expression of the IL-2 receptor.

The clinical importance of our finding both early and late post–T cell–depleted grafts remains to be determined. The patients studied in this series all achieved durable engraftment. Only two patients developed clinical evidence of GvHD. Each of these patients did produce increased levels of IL-2 early posttransplant. However, other patients without GvHD produced comparable levels. Current prospective analyses performed on patients receiving T cell–depleted or unfractionated marrow grafts as part of a randomized trial should determine whether our findings of early spontaneous IL-2 production and delayed regeneration of IL-2–responsive T cell functions are correlated with clinical events such as graft rejection, which complicate T cell–depleted grafts, or with other posttransplant complications such as recurrent infection or leukemia relapse.

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Risk of AIDS for Recipients of Blood Components From Donors Who Subsequently Developed AIDS

By Herbert A. Perkins, Susan Samson, Jane Garner, Dean Echenberg, James R. Allen, Morton Cowan, and Jay A. Levy

Reported cases of acquired immunodeficiency syndrome (AIDS) in San Francisco as of March 31, 1986, include 92 individuals who had donated blood subsequent to 1978. Their donated blood components had been transfused into 406 different recipients. The current status of 336 of these recipients was ascertained as of April 1, 1986. Of these, 223 had died at the time of our first contact, almost all as a result of the condition for which they were transfused. Seven had developed AIDS; five of these died, two before entry into the study and three subsequently. Forty-six additional living recipients were interviewed and evaluated. Seven had the AIDS-related complex, 18 had antibody to the human immunodeficiency virus (HIV) but were otherwise healthy, and 19 had no detectable anti-HIV. Two had risk factors other than transfusion. The frequency of infection of the recipient decreased as the time interval between transfusion and the diagnosis of AIDS in the donor increased. This information should be useful when counseling patients who have been transfused with blood components from donors later found to be infected with HIV.

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ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) is caused by a virus or a group of related viruses designated lymphadenopathy-associated virus[1] human T cell lymphotropic virus, type III,[2] or AIDS-associated retrovirus.[3] Recently an international committee has recommended that these viruses be named human immunodeficiency viruses (HIV).[4] HIV can be transmitted to patients by transfusion of blood components from donors who are carriers of the virus. Although most of these carrier donors appear healthy when subsequently examined, virus can usually be cultured from their peripheral blood lymphocytes.[5] Other HIV-infected donors have later developed symptoms, signs, and laboratory test results consistent with AIDS-related complex (ARC),[6] and a small proportion subsequently meet the criteria for a diagnosis of AIDS as defined by the Centers for Disease Control (CDC).[7] Before 1983 it was not recognized that persons from groups at high risk for AIDS should be excluded as blood donors, whereas it is now evident that substantial numbers of cases of AIDS occurred in this country as early as 1979, with a few cases occurring even earlier.

The three national blood bank organizations (American Association of Blood Banks, American Red Cross, and Council of Community Blood Centers) have endorsed a statement that recipients should be notified when a donor of a blood component that they have received subsequently develops AIDS.[8] Interviewers of patients with AIDS should always ask whether they had donated blood for transfusion. If so, the center where the blood was donated should be notified. Often this procedure is done through the local health department.

Ultimately the recipient is informed of the potential exposure, and the patient's first concern will be about the likelihood that he or she may develop AIDS. This report presents information on the risk of becoming infected with HIV and of developing AIDS for recipients of blood components whose donors subsequently develop AIDS. It is likely that the findings apply equally well when the donor remains healthy but anti-HIV is detected. The majority of donors with Western blot–confirmed anti-HIV (over 90% in our experience) have cells in their blood from which HIV can be cultured.

PATIENT SELECTION AND METHODS

The Irwin Memorial Blood Bank collects blood from volunteer donors in San Francisco and seven other counties of northern California. More than 100,000 donations have been given yearly for the past 7 years. As of March 31, 1986, 1,870 cases of AIDS had been reported to the San Francisco Department of Public Health, which has resulted in the highest per capita incidence of any major city in this country. The first case of AIDS associated with receipt of a blood transfusion was reported from San Francisco in December 1982.[9,10] The blood donor developed AIDS 9 months after the blood had been given. In December 1982, the Irwin Memorial Blood Bank and the San Francisco Department of Public Health agreed to share information so that they could identify all possible AIDS patients who had donated blood at an Irwin facility subsequent to 1978.

The list of AIDS patients reported to the San Francisco Department of Public Health was compared at regular intervals with the blood bank files to identify the dates of donation, the blood components made from each donation, and the hospitals to which the components were dispensed. The hospital contacted the physician who ordered the blood or the current personal physician of the recipient. The physician informed the patient that he or she may have been exposed to the AIDS agent through transfusion. The physician was also requested to ask his or her patient to participate in this current investigation. With permission of the personal physician, the recipient was contacted, provided with information about the study, and asked for written consent to be included in the study after the nature of the procedures had been fully explained. The protocol and the consent forms were approved by the University of California San Francisco Committee on Human Research.

Each consenting recipient was interveiwed by an experienced member of the blood bank epidemiology staff to determine whether there was any evidence that he or she had a risk factor for AIDS

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apart from transfusion. Such patients were eliminated from the final analysis. The interview, which included a brief physical examination, also searched for evidence of AIDS or ARC. Blood samples were taken for laboratory tests. T cell subset analysis was done in the first 13 cases by fluorescent microscopy using OKT4 and OKT8 monoclonal antibodies (Ortho Pharmaceutical Corp, Raritan, NJ) and in the remainder by flow cytometry using Leu-3 and Leu-2 monoclonal antibodies (Becton Dickinson, Mountain View, CA) in a Becton Dickinson FACS Analyzer. Mitogen stimulation of lymphocytes with phytohemagglutinin (PHA) was done in most cases. Mixed lymphocyte cultures (MLC) were attempted in the first 13 cases and stimulation with pokeweed mitogen (PWM) in the remainder. β_2-Microglobulin levels were measured by an enzyme immunoassay microtiter technique using reagents supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Commercially available Abbott kits (Abbott Laboratories, Abbott Park, IL) were used for the hepatitis tests, and anticytomegalovirus (CMV) antibody was detected by reverse passive latex agglutination (BBL Microbiology Systems, Cockeyesville, MD). Serological tests for antibody to HIV were performed by licensed enzyme immunoassay (EIA) methods at the blood bank. The Abbott EIA method was used in all cases; ENI (Electronucleonics, Columbia, MD) and Genetic Systems (Seattle, WA) methods also were used when there were discrepancies with other tests. Other tests for anti-HIV in all cases were the immunofluorescent antiglobulin slide test (IFA) at the University of California, San Francisco (UCSF)[3] and the immunoblot (IB) (Western blot) technique at CDC and UCSF.[11] Antibody was considered to be present if both IFA and IB were positive and to be absent if both IFA and IB were negative.

Although the majority of recipients had consistent results in their multiple tests for antibody to HIV, discrepancies between IFA and IB reports were initially noted in six of the 53 cases (11%). Criteria for the IB were not standardized initially, but later a gp41 band was required for a positive reaction, and with multiple blinded replicates, the two laboratories ultimately reported identical results.

RESULTS

Clinical status of the recipients. As of March 31, 1986, the San Francisco Department of Public Health list of 1,870 reported cases of AIDS meeting requirements of the CDC definition included 90 previous donors from the Irwin Memorial Blood Bank. Two additional donors from a neighboring blood bank were included early in the investigation; subsequently, only Irwin donors were followed. Most donations had been separated into components, each of which had been given to a different recipient, and most of the donors had given blood multiple times since 1978. The total number of patients who had received the components under investigation was 406. Of these, 223 (54.9%) were deceased at the time of our first contact with their physician. According to the records, almost all of these died as a result of the illness for which they were transfused, but two cases of AIDS had been recognized. In the rest, no evidence for HIV infection had been noted clinically or (when done) at autopsy, but it had rarely been considered in the differential diagnosis. The most common indications for transfusion of the deceased patients were gastrointestinal bleeding, leukemia, and cardiac surgery. The most common cause of death was leukemia, but death of unknown cause was equally common. The average interval between transfusion and death was 10 months. Of the remaining recipients, 113 (27.8%) were alive, and the status of 70 (17.2%) has not yet been determined. Information on the clinical status of the 223 dead and 113 living recipients was obtained from their personal physicians.

Seven of these recipients had developed AIDS, two died before initiation of this study and three between entry into this study and preparation of this report. Two were still alive at the time of this report. We interviewed and evaluated 46 additional recipients who were alive at the time of our initial contact. These were all of the living recipients who lived within several hundred miles of us and for whom permission had been obtained to participate in the study. Table 1 summarizes the data obtained concerning these 53 recipients. Eight of the recipients had signs, symptoms, and laboratory test results consistent with ARC.[6] The remaining 38 recipients appeared to be healthy, but 19 of them had antibody to HIV in their sera, and in the other 19 no antibody could be detected. The percentage of potentially exposed recipients for whom evidence of infection was obtained was thus 62% (33/53). Two of the living recipients (one with ARC and one with anti-HIV only) acknowledged having other risk factors for AIDS; therefore, they will be eliminated from further analysis. Case no. 14 had had repeated sexual contacts with a bisexual male whose antibody status is unknown. Case no. 33 gave a history of using drugs by injection.

Although there was a wide distribution of recipient ages in all four groups, of the four recipients aged 10 or less, one had AIDS, and three had ARC. Of the five recipients aged 80 or older, two had AIDS, and two had ARC. There was no clear effect of the sex of the recipient on susceptibility to infection or disease. Although 13 of the 31 infected individuals were female and only four of 20 noninfected individuals were female, the differences are not statistically significant. The most common indications for transfusion were cardiac surgery (20 cases), orthopedic surgery, (7 cases) and gastrointestinal bleeding (7 cases), all conditions that tend to require large amounts of blood. There was no obvious correlation between the condition prompting transfusion and the occurrence of infection or AIDS in the recipient, although the numbers are too small for meaningful statistical analysis. Diseases commonly associated with immunologic depression were not encountered among the living patients we investigated. It should be noted that they would not have been reported to us as AIDS cases because the CDC definition excluded them.

Among those patients who were alive when first seen, the death rate was highest in those with AIDS, was much lower in those with ARC and with antibody only, and was zero in those without antibody. These statements are based on the patient status when we first attempted to contact them. The numbers of RBC, platelet, and fresh frozen plasma components were all significantly greater for recipients who developed AIDS, but not if patient no. 3 is omitted. Patient no. 3 received many platelet transfusions and repeated therapeutic plasmapheresis for idiopathic thrombocytopenic purpura. HIV infection was not suspected in this case before the transfusions. There were no differences in the numbers of components transfused between those patients with ARC, those with antibody only, and those without detectable

Table 1. Recipients of Blood Components From Donors With Aids

Case No.	Age	Sex	Diagnosis	Status	AIDS	Antibody to HIV EIA	Antibody to HIV IFA	Antibody to HIV IB	Transfused RBC	Transfused PLT	Transfused FFP	Component From AIDS Donor	WBC	Lymph	H/S	PHA	PWM	MLC	Beta 2-M	HBsAg	Anti-HBc	Anti-HBs	Anti-CMV
1	1	M	Hem Dis NB	Dead	AIDS			0	22	8	0	PLT	7.7						5.9				0
2	80	M	Cardiac Surg	Dead	AIDS	+	+	+	5	0	2	RBC	4.1	0.4	0.3	10,473			16.5	0	0	0	+
3	52	M	Thromb Purp	Alive	AIDS	+	+	+	31	128	362	PLT	3.8		0.1	1,253	560		5.1	0	0	0	0
4	56	F	Cardiac Surg	Alive	AIDS	+	+	+	17	18	12	PLT	7.7	3.3	0.4	3,380	3,800		10.0	0	0	0	+
5	81	F	Orthop Surg	Dead	AIDS	+	0	0	2	0	0	RBC			0.1	34,096	4,688		6.0	0	0	+	+
6	23	M	Trauma	Dead	AIDS		+		2	0	0	RBC			0.5								
7	50	F	Orthop Surg	Dead	AIDS	+	+	+	13	21	2	PLT	1.8	0.3		16,600		2,270	4.9				
8	10	M	Orthop Surg	Alive	ARC	+	+	+	10	7	0	PLT	6.4	2.6	0.5	13,290		7,040	1.8	0	0	0	+
9	1	F	Plastic Surg	Alive	ARC	+	+	+	1	0	0	RBC	5.7	4.1	1.7	28,890			2.1	0	0	0	+
10	89	F	CA kidney	Dead	ARC	+	+	+	7	0	0	RBC	4.8	1.5	0.3	87	0		15.0	0	0	0	+
11	87	F	GI bleed	Dead	ARC	+	+	+	7	0	0	RBC	3.6	1.0			4,477		13.5	+	0	0	+
12	68	M	Cardiac Surg	Alive	ARC	+	+	+	7	8	4	FFP	3.8	0.8	0.1	295	240		2.5	0	0	0	0
13	4	M	Resp distress	Alive	ARC	+	+	+	12	0	1	FFP			0.9	951	1,398		4.8	0	0	0	0
15	67	F	Orthop Surg	Alive	ARC	+	+	+	2	0	0	RBC			0.4				4.7	0	0	0	+
16	29	F	Eclampsia	Alive	Ab+	+	+	+	19	9	0	PLT				31,262	20,482		3.0	0	0	0	+
17	40	F	Miscarriage	Alive	Ab+	+	+	+	2	0	0	RBC	6.1	1.5	0.7	18,748	15,829		2.1	0	+	0	+
18	80	F	Orthop Surg	Alive	Ab+	+	+	+	3	0	0	RBC			1.6	44,590		21,789	2.4				
19	42	F	Gyn Surg	Alive	Ab+	+	+	+	1	0	0	RBC			0.7				3.6	0	0	0	+
20	51	M	Cardiac Surg	Alive	Ab+	+	+	+	9	10	6	PLT	4.3	1.6	0.7	8,374	447		3.0	0	0	0	+
21	50	M	GI bleed	Dead	Ab+	+	+	+	19	0	0	RBC	3.6	1.4		25,010		7,860	5.3				
22	61	M	Cardiac surg	Alive	Ab+	+	+	+	12	20	6	RBC			0.6	758	3,611		5.3	0	+	0	+
23	42	M	Cardiac surg	Alive	Ab+	+	+	+	10	10	3	RBC			0.7	7,529	3,518		2.8	0	0	0	0
24	61	M	GI bleed	Alive	Ab+	+	?	+	3	0	0	RBC	6.7	1.4	0.8				5.5	0	0	0	+
25	46	F	GI bleed	Alive	Ab+	+	+	+	8	10	5	PLT	5.7	1.9	0.3	2,014	375		4.8	0	0	0	+
26	38	M	Gen Surg	Alive	Ab+	+	+	+	2	0	0	RBC	7.7	2.5	0.5	2,141	285		3.6	0	0	0	+
27	62	M	Cardiac Surg	Alive	Ab+	+	+	+	9	0	0	RBC	3.0	1.0	0.7	9,655	15,009		2.2	0	0	0	+

28	72	F	Arthritis	Alive	Ab+	+	+	+	2	0	0	RBC			2.5	13,449	13,386		5.7	0	0	0	+
29	77	M	Cardiac Surg	Dead	Ab+	+	+	+	8	0	0	RBC	4.6	2.8					5.6	0	0	0	+
30	27	M	GI bleed	Alive	Ab+	+	+	+	10	0	0	RBC	4.8	1.6	0.7	6,805	885		3.8	0	+	+	+
31	60	M	Cardiac Surg	Alive	Ab+	+	+	+	8	0	0	RBC	5.5	1.9	0.4	11,130		10,800	2.9				
32	59	M	Cardiac Surg	Alive	Ab+	+	+	+	4	10	2	RBC			0.2	1,088	312		3.0				
34	70	F	Gen Surg	Alive	Neg	0	0	0	14	5	1	PLT			1.3	10,690		9,490	6.9				
35	50	M	Cardiac Surg	Alive	Neg	0	0	0	3	0	0	RBC	7.6	1.7	2.0	32,400		1,665	2.2	0	0	0	+
36	29	M	Polycyst Kid	Alive	Neg	+	0	0	5	0	0	RBC	3.9	1.1	0.1	144	149		6.1	0	0	0	+
37	65	F	Vasc Surg	Alive	Neg	0	0	0	10	10	0	R/P			0.5	21,611	4,343		2.5	0	0	0	+
38	52	M	Cardiac Surg	Alive	Neg	0	0	0	6	0	1	RBC	4.3	1.5	1.1		3,685		1.2	0	0	0	+
39	66	F	Orthop Surg	Alive	Neg	0	0	0	3	0	0	RBC	3.6	1.4	2.8		2,379		4.0	0	0	0	+
40	70	M	Vasc Surg	Alive	Neg	0	0	0	25	6	4	PLT	7.4	1.5	1.4				5.5	0	+	+	+
41	71	M	Cardiac Surg	Alive	Neg	0	0	0	3	0	0	RBC			1.6				1.5	0	+	0	+
42	60	M	Cardiac Surg	Alive	Neg	0	0	0	7	10	4	RBC	8.1	2.4	2.0	32,930		2,440	2.8				
43	76	F	Orthop Surg	Alive	Neg	0	0	0	2	0	0	RBC			0.9	16,846	7,622		9.2	0	0	+	+
44	74	M	Cardiac Surg	Alive	Neg	0	0	0	12	20	5	PLT	6.6	1.9	1.0	11,618	7,890		2.3	0	0	0	+
45	70	M	Cardiac Surg	Alive	Neg	0	0	0	10	10	8	FFP	8.8	2.9	1.6				1.4	0	+	+	+
46	27	M	Fracture Fem	Alive	Neg	0	0	0	14	0	0	RBC	5.6	2.3	0.5	6,006	2,204		1.3	0	0	0	+
47	70	M	Cardiac Surg	Alive	Neg	0	0	0	10	10	4	PLT	4.9	1.0	2.4	28,900		23,400	2.0				
48	72	M	Cardiac Surg	Alive	Neg	0	0	0	8	0	0	RBC	8.1	2.0	1.7	22,142	29,358		3.4	0	0	0	+
49	68	M	Trauma	Alive	Neg	0	0	0	8	0	0	RBC	8.6	2.3	3.2	11,132	14,179		1.5	0	0	0	0
50	40	M	Cardiac Surg	Alive	Neg	0	0	0	6	5	0	PLT	4.5	2.6	2.1	21,500		15,360	1.0				
51	50	M	GI surgery	Alive	Neg	0	0	0	6	0	0	RBC	6.7	2.4	1.3	25,725		2,414	1.2				
52	77	M	GI bleed	Alive	Neg	0	0	0	3	0	0	RBC	6.7	2.5	0.8		1,217		2.9	0	0	0	+
53	64	M	GI bleed	Alive	Neg	0	0	0	13	0	2	FFP	6.9	3.1	2.1				2.4	0	0	0	+

Abbreviations: RBC, red blood cells including whole blood; PLT, platelets; FFP, fresh frozen plasma; R/P, RBC and platelets; WBC, white blood cell count per microliter × 1,000 (4.0 to 10.0)*; Lymph, lymphocyte count per microliter × 1,000 (1.5 to 4.0)*; H/S, helper/suppressor ratio (>1.0)*; PHA, phytohemagglutinin stimulation ($>11,500$ cpm)*; PWM, pokeweed mitogen stimulation ($>3,000$ cpm)*; MLC, mixed lymphocyte culture ($>5,000$ cpm)*; Beta 2-M, β_2-microglobulin (<2.6 mg/dL)*; Hem Dis NB, hemolytic disease of the newborn; Thromb Purp, thrombocytopenic purpura; Surg, surgery; Orthop, orthopedic; CA, cancer; GI, gastrointestinal; Resp, respiratory; Vasc, vascular; Fem, femoral bone.

*Normal range.

antiviral antibody. All three types of components had been provided by the donors who later developed AIDS. RBC had been given to 21 infected recipients and 13 not infected; platelets to eight and seven, respectively; and fresh frozen plasma to two each.

Table 2 relates the recipient's chance of infection or disease to the time interval between the transfusion and the diagnosis of AIDS in the donor. In six of the seven cases where the recipient developed AIDS, the donor was recognized to have AIDS within 22 months. Infection of the recipient was apparent in 90% of the cases where the donor developed AIDS within a year, in 62% when the donor's AIDS was recognized between 1 and 4 years, in 20% of those with an interval between 4 and 5 years, and in neither of the two cases where the interval was over 5 years. Although it seems obvious that the longer the interval between transfusion and the diagnosis of AIDS in the donor, the less likely the donor was infected when the blood was taken, it should be noted that the differences in Table 2 lack statistical significance.

Laboratory data. Of the seven cases of AIDS, two had no detectable antibody by IB. Case no. 1 (our original case of transfusion-associated AIDS)[10] was tested by IB alone. Case no. 5 was positive in the Abbott EIA, negative by Genetic Systems EIA, and negative by IFA and IB and was therefore considered to be a false-positive by the Abbott method. All tests on the ARC cases were consistently positive. In case no. 36 three EIA tests were reactive (Abbott, ENI, and Genetic Systems), but both IFA and IB were nonreactive. Based on the latter two test results and the known propensity of EIA tests to result in false-positives, this result is assumed also to be an EIA false-positive.

A low WBC count was more common in the AIDS and ARC groups. The absolute lymphocyte count was progressively lower from the antibody-negative to the antibody-positive groups to those with ARC to those with AIDS, but it is noteworthy that three of the 15 measured in the antibody-negative group (20%) had levels below 1,500/μL. Helper/suppressor ratios were below 1.0 in all of the AIDS cases and in more than 80% of those with ARC or with anti-HIV antibody alone. They were also low in 25% of those who were negative for anti-HIV antibody. Stimulation indexes with PHA, PWM, and MLC did not correlate with the category into which the patient fell. β_2-Microglobulin levels were high in all of the AIDS cases, but they were abnormal in more recipients who had anti-HIV antibody alone than in those with ARC. Moreover, 40% of those negative for antiviral antibody had an elevated β_2-microglobulin level.

Table 2. Effect of the Time Between Blood Donation and the Development of AIDS in the Donor on the Risk of Infection in the Transfused Recipient

Months	AIDS	ARC	Asymptomatic With Anti-HIV	No Anti-HIV
0-12	4	2	3	1
13-24	2	1	4	7
25-36	0	1	6	4
37-48	1	3	3	2
49-60	0	0	2	3
>60	0	0	0	2

The laboratory tests were performed at a mean interval of 26 months after transfusion (range, 10 to 43) in the patients with AIDS, 38 months (range, 12 to 71) with ARC, 32 months (range, 9 to 60) with anti-HIV only, and 43 months (23 to 78) in the patients with no antibody to the virus detected.

DISCUSSION

Our observations on previous recipients of blood components from donors who later developed AIDS demonstrate that these recipients are at relatively high risk of developing AIDS themselves or of being infected by HIV. Of 336 recipients whose current status is known, seven (2%) have developed AIDS. Of 46 living recipients who do not have AIDS, 24 have antibody to HIV (52%), and seven of these meet the criteria for ARC.

The blood components transfused from the donors who developed AIDS included all routine components. This fact confirms the prevous CDC report that AIDS can be transmitted by whole blood, RBC and platelet concentrates, and fresh frozen plasma.[12] There is no evidence in this small series that any of these components is more likely to transmit AIDS than the others.

Infection occurred after transfusion of even a single unit (case nos. 9 and 19), but in general our patients received relatively large amounts of blood components. Recipients who developed AIDS received more components on the average than those with ARC, with anti-HIV only, or with no evidence for infection; but the difference is not significant if the atypical case 3 is omitted. Curran et al[12] have already shown that recipients with AIDS tended to receive large numbers of blood components and have reasoned that the more donors to whom a recipient is exposed, the greater the possibility of receiving an infected unit. This is undoubtedly true, but in contrast to the study of Curran et al where the recipients were selected because they had developed AIDS, the recipients in our study were selected because they had received a component from a donor who developed AIDS. Moreover, we have to explain why antibody-negative recipients had received an equally large number of components. The most likely explanation for our results is that a single specified component, no matter how selected, is more likely to have been given to a patient who received large numbers of components than to a recipient of one or two components. Whatever the explanation, the relatively large number of components given to these patients could act as a cofactor for the development of AIDS in view of the well-established immunosuppressive effects of blood transfusion.[13]

That two thirds of the recipients we identified were already dead when we first attempted to contact them can also be explained by our case selection method and should not be interpreted to be the typical fate of all transfusion recipients. Patients who receive large amounts of blood are more likely to have conditions that result in early demise. There was no evidence in the medical records that this excess of early deaths was related to AIDS. Our assumption that they were an artifact of the method of case selection requires comparison with a control group of recipients selected by identifying a donor who was not infected with HIV. Such control studies are underway.

The absolute lymphocyte count and, to a lesser degree, the total WBC count were lowest in AIDS cases compared with those with ARC, were less often low in antibody-only cases, and were most often normal in the antibody-negative group. Helper-suppressor ratios were low in all AIDS cases, in 80% of antibody-positive cases (whether or not ARC was present), but also in 25% of antibody-negative cases. Obviously, in this group of subjects ill enough to require previous transfusions, a low helper-suppressor ratio is even less indicative of AIDS or HIV infection than it is in a healthier population. Functional lymphocyte stimulation tests showed a very poor correlation with the presence or absence of HIV infection or the stage of disease and would appear to be of no help in this type of patient who has other reasons for abnormal results in these procedures. β_2-Microglobulin, which has been suggested as a test that might indicate the likelihood of overt AIDS occurring,[14] also appears to be of little help in this situation because of the high frequency of abnormal results in patients with no evidence of infection by the AIDS virus, presumably a result of coexisting illnesses.

Hepatitis B tests were usually negative in these patients, in contrast to their high rate of positive results among the more common high-risk groups for AIDS. Homosexually active males and intravenous drug users are very likely to have been exposed to hepatitis B virus. Blood transfusion recipients are exposed far less often because all donor blood is tested for hepatitis B antigen, and positive units are discarded. In contrast to the results with hepatitis B tests, our blood recipents had a very high frequency of antibody to CMV (34/40, or 85%) compared with the 51% rate of positive results among our blood donors. This finding suggests that exposure to CMV is very likely to occur during blood transfusions, but the numbers are small, and the recipients were generally older than most blood donors. The frequency of anti-CMV increases with the age of the subject. It is noteworthy that the frequency of anti-CMV antibody did not correlate with the presence or extent of HIV infection in view of the common hypothesis that infection with CMV may be a cofactor that increases susceptibility to the AIDS virus.[15]

The most useful prognostic indicators for these recipients were the time interval from transfusion until the donor was diagnosed as having AIDS and the age of the recipient. Recipients below age 11 or over age 79 were much more likely to develop overt disease than those of intermediate ages. This latter finding may reflect the state of the immune system at these ages. The shorter the time interval between the transfusion and the onset of AIDS in the donor, the more likely the donor was to be infected already at the time the blood was given, and the more likely, therefore, that the recipient would have antibody as a sign of infection. Moreover, the occurrence of disease as well as infection appears to be more likely the shorter the interval (Table 2).

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Limiting-Dilution Analysis of the Effects of Colony-Stimulating Factors, Phytohemagglutinin, and Hydrocortisone on Hematopoietic Progenitor Cell Growth

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The effects of colony-stimulating factors (CSFs), phytohemagglutinin (PHA), and hydrocortisone on the growth of human bone marrow hematopoietic progenitor cells (granulocyte-macrophage; GM) were analyzed in a limiting-dilution assay (LDA). Both low-density bone marrow cells separated by discontinuous Percoll gradients and a T cell–depleted and progenitor-enriched cell fraction obtained by the combination of counterflow elutriation centrifugation and Percoll gradients were examined in LDA. GCT (monocytoid cell line–conditioned medium containing GM-CSF), human placenta-conditioned medium, bladder carcinoma cell line 5637–conditioned medium (containing GM- and G-CSF), and recombinant CSF (G-CSF) directly induced proliferation of progenitors with single-hit kinetics. In some instances, however, PHA-stimulated lymphocyte-conditioned medium (containing G- and GM-CSF) showed deviation from single-hit kinetics, which demonstrated the presence of factor(s) suppressive to progenitor growth. In a T cell–depleted, progenitor-enriched fraction, PHA alone was found to suppress progenitor growth at a level as low as 100 ng/mL. The addition of hydrocortisone (10^{-6} mol/L) increased the progenitor frequency but suppressed progenitor growth at 10^{-4} mol/L. LDA appears to be a valuable method for exploring mechanisms of factors regulating hematopoietic cell growth.

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GRANULOCYTE-MACROPHAGE (GM) colony-stimulating factors (CSFs) are glycoproteins that regulate growth and differentiation of GM progenitors.[1] Agar and methylcellulose colony assays have been used to evaluate the effects of CSF,[2,3] to monitor purification of committed hematopoietic progenitors,[4-10] and to represent a correlate of hematopoietic restoration potential of bone marrow (BM) aspirates.

Colony assays in semisolid media have aided the understanding of hematopoiesis, but they have limitations. The different preparations of CSFs used and the criteria used to define clonal growth make colony results from various laboratories difficult to compare. Agar or methylcellulose may disrupt cellular and extracellular matrix interactions that play a role in hematopoietic proliferation and differentiation.[11,12] These assays are not linear except at relatively high cell concentrations,[13-15] and under these conditions, the number of colonies obtained may be influenced by helper or suppressor accessory cell functions. To conclude that a factor has a direct effect on progenitors, it should be demonstrated that the observed colony growth is based on single-hit kinetics (ie, the factor is affecting only one cell type and not operating via an accessory cell). In these culture systems, assessment of the effect of CSFs on progenitor growth requires purified progenitors.[16] However, human hematopoietic progenitor purification is time and labor intensive and has met with only limited success.[17]

Limiting-dilution analysis has been used to enumerate murine hematopoietic progenitors,[18,19] and recently, we have developed a quantitative assay of human BM GM progenitors by limiting-dilution assay (LDA).[20] This method was shown to detect approximately three times more total progenitors than methylcellulose colony assays. LDA offers the potential for examining mechanisms of factors regulating progenitor growth in vitro and, hence, is an ideal method for resolving several of the aforementioned problems. Using this method, we have evaluated the stimulatory activities of CSFs on progenitor growth by using low-density BM cell fractions from Percoll gradients. The effects of phytohemagglutinin (PHA) and hydrocortisone (HC) on progenitor growth were also analyzed with a T cell–depleted and progenitor-enriched fraction that was obtained by the combination of counterflow elutriation centrifugation (elutriation)[21] and Percoll gradient separation.[22] We have found that a human giant cell tumor line (GCT), human placenta–conditioned medium (HPCM), bladder carcinoma–conditioned medium (BCM), and recombinant CSF can stimulate the growth of hematopoietic progenitors independent of the effects of accessory cells. PHA suppressed CSF-stimulated progenitor growth, and HC increased the progenitor frequency at 10^{-6} mol/L. This analysis allows evaluation of various agents that affect hematopoietic cell growth in vitro.

MATERIALS AND METHODS

BM Cell Fractionation

BM aspirates were fractionated to enrich for mononuclear cells as previously described.[22] Without this enrichment, it was difficult to detect colonies because of the greater number of nonproliferating cells. Briefly, BM was aspirated from normal donors after obtaining informed consent. Cells were centrifuged at 600 g for 25 minutes, and buffy coat cells (BMBC) were collected. A stock solution of 100% Percoll was prepared by diluting Percoll (Sigma Chemical Co, St Louis) with 1/10 volume of 10× phosphate-buffered saline (PBS). The 100% solution was then diluted with Dulbecco's PBS (GIBCO, Grand Island, NY) to obtain 60% and 40% Percoll

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solutions. BMBC were layered over a 8-mL gradient consisting of 4-mL layers of 60% and 40% Percoll (Sigma) in 15-mL polystyrene tubes (Becton Dickinson, Oxnard, CA). These were centrifuged for 20 minutes at 750 g. Cells in the 40/60 Percoll interphase were aspirated and washed twice with Hank's balanced salt solution supplemented with 1% fetal bovine serum (FBS). The 40/60 Percoll fraction is depleted of erythrocytes, granulocytes, platelets, and nucleated red cells and enriched in immature blasts.[22] Cells thus obtained (Fr 40/60) were used to study the effect of CSFs on progenitor growth.

LDA of Progenitors

LDA was performed as previously described.[20] Briefly, BM cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.6×10^{-2} mol/L $NaHCO_3$, 1.8×10^{-2} mol/L HEPES, 3×10^{-5} mol/L hypoxanthine, 3×10^{-6} mol/L thymidine, 10^{-3} mol/L pyruvate, 5×10^{-5} mol/L 2-mercaptoethanol, 10^{-4} mol/L minimal essential medium (MEM) nonessential amino acids (GIBCO), 15% FBS, and CSF at various concentrations. For the study of CSFs, 10^{-6} mol/L HC sodium succinate (Elkins-Sinn, Inc, Cherry Hill, NJ) was added to the medium to suppress the growth of residual T cell clones. The cell suspension was mixed by pipetting and placed in 60-well microplates (Flow Laboratories, McLean, VA) at seven to nine cell densities (10 to 400 cells/10 μL/well). Each dilution consisted of one or two plates (60 to 120 wells). The plates were then incubated in a humidified atmosphere (37°C, 5% CO_2 in air), and the medium was replaced every other day. After 10 days the number of cells in the largest cluster of blasts in each well was counted by using an inverted microscope. Control wells were incubated without CSFs.

Statistical Analysis

Linear regression analysis was applied to the limiting dilution data to calculate the best slope.[23,24] The resulting straight line allowed calculation of the progenitor frequency. Confidence intervals for the slope were calculated by using a form of Student's *t* test. Conformance of the data to linearity was tested by using a chi-square analysis; a probability $P > .05$ indicated single-hit kinetics, thus demonstrating the activity of a single limiting cell type.

Morphological Evaluation

The cells were examined morphologically by using Wright's stained cytocentrifuge preparations (Shandon Products, Astmoor, England). To minimize distortions, slides were also made by unit gravity sedimentation. This was especially useful in examination of immature blasts.[25] The slides were also analyzed for nonspecific esterase, naphthol-AS-D-chloroacetate esterase, and myeloperoxidase (Sigma) by following the manufacturer's instructions.

CSFs

Five types of CSFs were evaluated at various concentrations. HPCM, prepared by the method of Burgess et al,[26] was tested at 1% to 20%. PHA-stimulated leukocyte-conditioned medium (PHA-LCM) was prepared by using the method of Iscove et al[27] with minor modifications. Normal donor peripheral mononuclear cells were cultured at 2×10^6 cells/mL in DMEM with 7.5% FBS and 7.5% human AB serum in the presence of PHA (10 μg/mL, Sigma) at 37°C, 5% CO_2 for four days. The medium was harvested, filtered, and tested at 1% to 10%, with and without 1 U/mL erythropoietin (Epo; Connaught, Toronto, Ontario, Canada). A single lot of the culture medium of GCT[28] (GIBCO) was used at 1% to 20%. Conditioned medium of human bladder carcinoma cell line 5637 (kindly provided by Dr Fogh, Memorial Sloan-Kettering Cancer Center)[29] was tested at 0.5% to 10%. The 5637 cells were cultured in RPMI 1640 supplemented with 10% FBS, and the medium was harvested every three or four days, filtered, and stored at 4°C. Recombinant CSF, provided by Dr Souza, Amgen, Inc (Thousand Oaks, CA), was tested at 10 to 500 U/mL.

Effects of PHA and HC on Progenitor Growth

PHA-M (Sigma) was diluted with supplemented DMEM. HC sodium succinate was prepared according to the manufacturer's instructions and then diluted with supplemented DMEM. The LDA was performed as earlier by using cells from elutriation and Percoll gradient centrifugation (Fr 3:40/60 cells) with the addition of PHA or HC. When testing PHA, no HC was added to the culture. Fresh PHA (0.01 to 10 μg/mL) and HC (10^{-9} to 10^{-4} mol/L) were prepared for each experiment.

RESULTS

Composition of Cells After Percoll Separation and Elutriation of BMBC

The cell recovery in Fr 40/60 from the Percoll gradient was 11.2% of the initial BMBC layer. The average cell composition in this fraction was in agreement with our previous study[22]: 18.7% unclassified blasts plus myeloblasts, 2.4% promyelocytes, 3.7% myelocytes plus metamyelocytes, 15.4% granulocytes, 9.1% erythroblasts, 13.7% monocytes or macrophages, and 39% lymphocytes.

For testing T cell–active agents for their direct effects on progenitors, T cells were eliminated by elutriation of BMBC cells before the Percoll gradient. After elutriation, the highest cell count was observed in the large cell fraction (Fr3), which contained about 60% of the input cells, most of them having the morphology of myelomonocytic cells. When this fraction was further separated by discontinuous Percoll gradient centrifugation, the 40%/60% layer (Fr: 3,40/60) contained 50% to 80% blasts and promyelocytes and was five- to ninefold enriched for progenitor activities over the BMBC layer.[21] By limiting-dilution analysis, we have shown

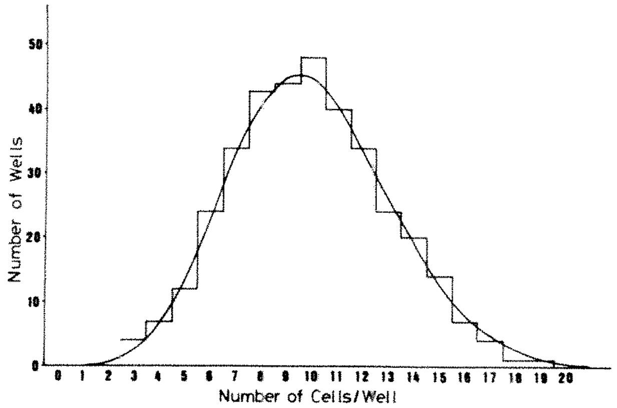

Fig 1. The actual distribution of cells in each well (bars) and the expected Poisson distribution (line) were shown. In this experiment, cells were plated at 10 cells/well in 360 wells, and three hours ater, the number of cells in each well was counted by using an inverted microscope. In a separate experiment, the actual distribution of cells at one cell/well was also tested. In both experiments, the observed distribution conformed to the Poisson distribution.

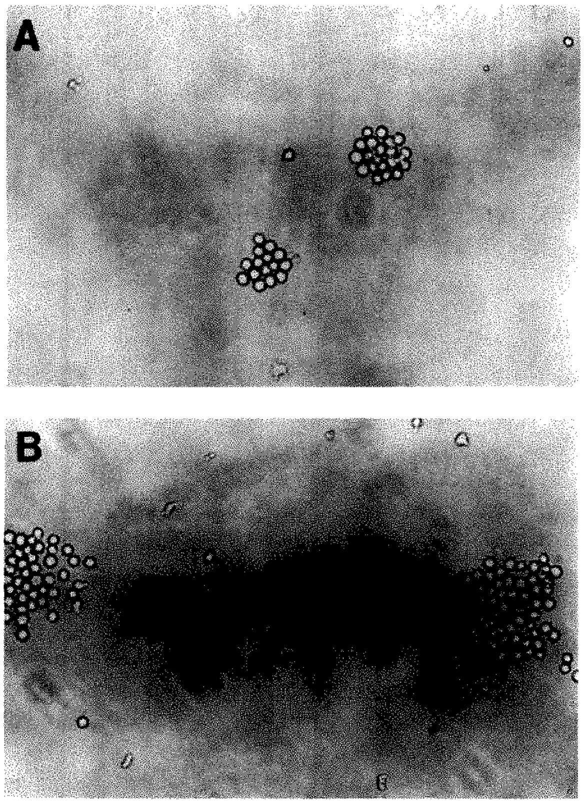

Fig 2. Examples of colonies in microwells examined by phase-contrast microscopy at day 10 of culture: (A) ≥10 cells, and (B) ≥40 cells colonies.

that this fraction contains only 1/3,000 to 1/10,000 T cells.[21]

LDA of the Growth of BM Cells

For the statistical approaches used in this analysis to be valid, it was necessary to demonstrate that the cell suspension was uniform (ie, did not contain clumps that would be scored as colonies) and could be distributed in the 10-μL wells by filling the plates (ie, the cells did not sediment into the wells from the additional volume above the wells during the plating). We verified the actual number of cells remaining in each well after distributing a known cell concentration into the microwell plates (Fig 1). In this experiment, the cells were plated at 1,000 cells/mL, and a typical Poisson distribution[30] was confirmed with a peak at ten cells per well. Cells were also plated at 100 cells/mL in 180 wells, and the cell distribution was examined in the same way; the percentage of wells that contained no cells was 36% (66/180). This is close to the result predicted by the zero term of the Poisson distribution (37% negative wells). Thus, the statistical vehicles used for the analyses were appropriate.

Clones of proliferating cells were distinguished from the nondividing cells remaining in the wells by their larger size, refractile surface by phase-contrast microscopy, and homogeneous appearance (Fig 2). Nonproliferating cells and aggregates usually disappeared by the seventh day of culture. We also investigated the optimum duration of growth for the analysis. The data obtained at day 10 showed the highest progenitor frequencies with narrowest confidence intervals in the linear regression analysis; therefore, cultures were scored on day 10. By day 14, the cells had usually degenerated, and colonies could not be scored.

Before determining the degree to which various factors influence growth of progenitors in vitro, it was necessary to establish the criteria to be used in scoring a well as positive for growth. No colonies consisting of more than eight cells were formed in wells with Fr 40/60 cells cultured without CSF or in wells with 500 purified T lymphocytes. Initially, assays were scored using multiple criteria (≥8, ≥10, ≥20, ≥30, and ≥40 cells/colony). When more than one colony was present, only the largest colony in each well was scored. When the confidence intervals were compared for each criteria, the criterion ≥10 cells was determined to be the most reliable. Calculated frequencies of progenitors cultured with 10% GCT and scored by the different criteria from eight different donors are shown in Table 1.

The estimated frequency of progenitor cells was obtained by analyzing the relationship between the number of cells plated per well and the percentage of wells found to be negative for cell growth. According to the zero term of the Poisson distribution, each well may be considered to contain an average of one precursor when 37% of a series of replicate wells are negative. In previous experiments using 10% GCT, it was shown that each criterion including ≥8, ≥10, ≥20, ≥30, and ≥40 cells/colony demonstrated single-hit kinetics,

Table 1. GM Progenitor Frequencies in LDA

	Number of Cells/Colony*				
Experiment	≥8	≥10	≥20	≥30	≥40
1	1/58 ± 4	1/72 ± 2	1/110 ± 17	1/147 ± 21	1/206 ± 29
2	1/56 ± 5	1/65 ± 1	1/123 ± 10	1/207 ± 17	1/290 ± 45
3	1/84 ± 5	1/99 ± 4	1/144 ± 6	1/202 ± 15	1/247 ± 18
4	1/115 ± 14	1/143 ± 12	1/249 ± 32	ND	1/353 ± 57
5	1/32 ± 4	1/39 ± 3	1/51 ± 12	1/84 ± 20	1/97 ± 9
6	1/74 ± 5	1/86 ± 4	1/124 ± 8	1/166 ± 14	1/203 ± 13
7	1/52 ± 7	1/59 ± 6	1/78 ± 11	1/88 ± 8	1/99 ± 10
8	1/40 ± 4	1/44 ± 2	1/47 ± 7	1/58 ± 11	1/71 ± 10
Mean	1/64	1/76	1/116	1/136	1/196

Abbreviation: ND, not determined.

*In each experiment, the same plates were scored five times by using criteria from ≥8 to ≥40 cells to define a colony. Precursor frequencies were determined as described in the test for each criterion. Data are given as the mean precursor frequencies for each criterion ± SD.

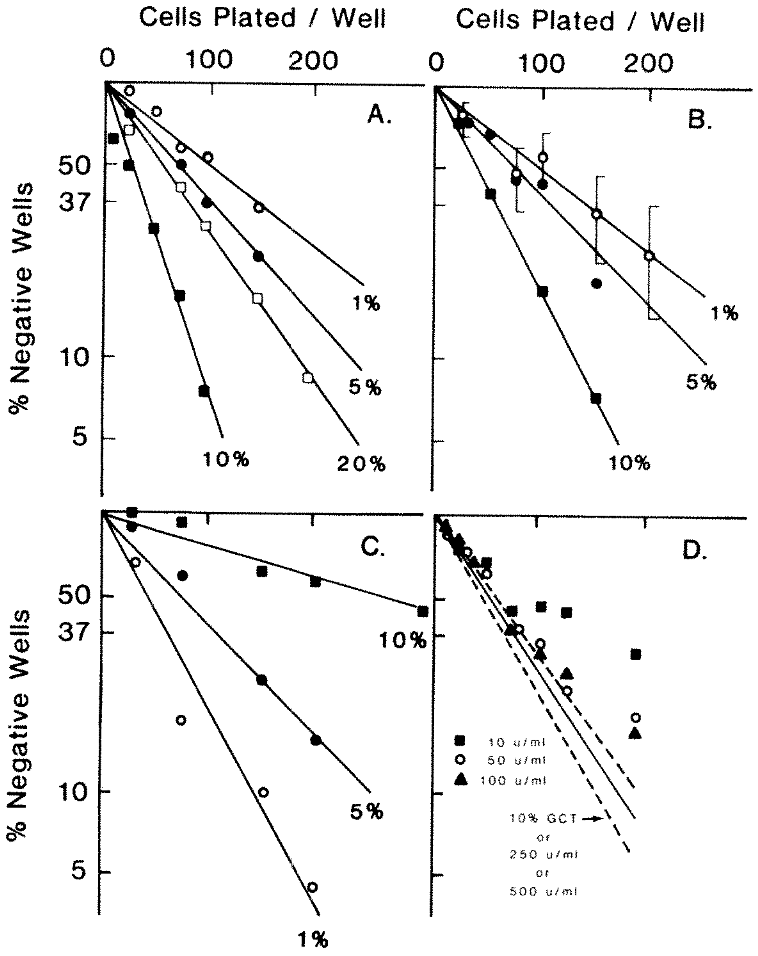

Fig 3. Representative results obtained from LDA of progenitors with CSFs. Each CSF was tested with three to four different donors. (A) GCT. A nonlinear response was observed in one of four donors at 1%. (B) HPCM. A nonlinear response was observed in one of four patients at 1%. (C) BCM. Nonlinear responses were observed in one of three donors at 0.5% and one of three patients at 10%. (D) Recombinant CSF: Approximately the same progenitor frequency was obtained with either 250 or 500 U/mL. Both of these results were within the confidence intervals for a parallel assay with 10% GCT. For clarity, only one line is shown. The progenitor frequency was slightly decreased with 50 and 100 U/mL, and single-hit kinetics were not observed with 10 U/mL in three donors.

thereby indicating that only one cell type was limiting in the culture system. Thus, no helper or suppressor accessory cell function affecting progenitor growth was observed under these conditions.

Screening of CSFs on Progenitor Growth

GCT. In the absence of a source of CSF, no colonies were observed. In most experiments, GCT supported progenitor cell growth with single-hit kinetics at various concentrations (1% to 20%); however, the highest frequency was observed with a concentration of 10%. The dose response of progenitor growth demonstrated normal biologic variation from donor to donor, but a representative experiment is demonstrated in Fig 3A. When progenitor frequency was compared with that of other CSFs, GCT usually yielded the highest frequency (Table 2). The colony-forming cells harvested from cultures containing GCT showed both myeloid (86%) and macrophage-monocyte (14%) differentiation (Table 3).

HPCM. A similar trend was observed in cultures containing HPCM. Progenitor growth with single-hit kinetics was supported by HPCM, and maximum progenitor yields were observed at 5% or 10% concentration (Fig 3B). Lower

Table 2. Progenitor Frequencies in LDA Cultured With Different CSFs

Experiment	10% GCT	10% HPCM	5% PHA-LCM	1% BCM
1	1/99	1/124	ND	ND
2	1/143	1/176	1/225	ND
3	1/39	1/167	ND	ND
4	1/59	1/57	1/118	1/99
5	1/44	1/28	1/71	1/57
6	1/132	ND	ND	1/171

In each experiment, cells from a single marrow sample were stimulated with the indicted sources of CSFs, and the progenitor frequencies were determined as described in the text by using the criterion ≥10 cells/colony.

Table 3. Effect of Different CSFs on Progenitor Maturation

	Differential Counts (%)			
Factor	Promye/Blast*	Juvenile	Polys	Macrophage/Monocyte
GCT	50	14.5	21.3	14.3
HPCM	75	9.1	11.2	4.4
PHA-LCM	53	11	8.3	28
BCM	55	8.2	20.3	16.3

BM mononuclear cells were stimulated with various sources of CSF as described in the footnote to Table 2, representative wells were harvested at day 10 and pooled, and differential morphological examinations were performed. The average results of three to four experiments are shown.

*Promye/Blasts include immature blasts, myeloblasts and promyelocytes. Juvenile myeloid cells include myelocytes and metamyelocytes. Polys are polymorphonuclear leukocytes.

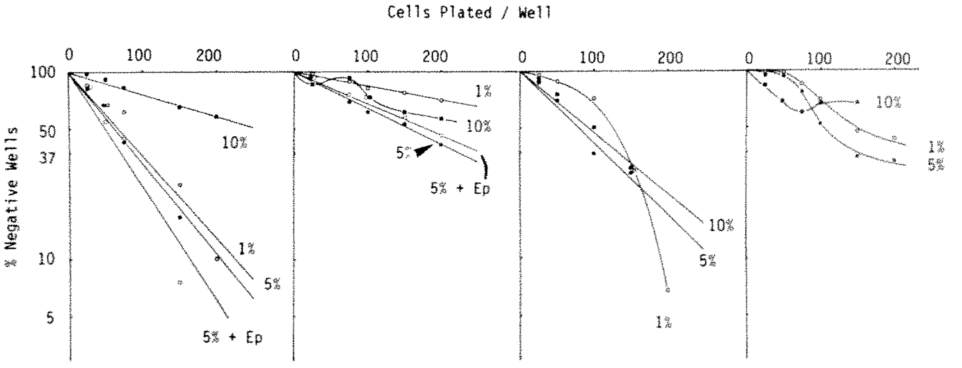

Fig 4. LDA of progenitors with PHA-LCM. Derivation from linearity were observed in several experiments.

levels appeared to be limiting, and in one donor, the response to 1% HPCM deviated from linearity.

BCM. Cell growth with single-hit kinetics was also usually observed with BCM (Fig 3C). The maximum progenitor yield was always obtained with 1% BCM. A nonlinear response was seen in one donor at the lowest concentration (0.5%) and in one donor at the highest concentration (10%). Because of the ability of a 1% concentration of BCM to yield about the same progenitor frequency obtained with other CSFs, the synergistic effect with GCT was evaluated by adding 1% BCM to 10% GCT in the culture. The progenitor yield remained almost the same as with GCT alone (data not shown).

Recombinant CSF. In three experiments, cultures containing 250 or 500 U/mL recombinant CSF yielded a linear response with approximately the same frequency of progenitors as 10% GCT. A slightly decreased frequency was obtained at 50 and 100 U/mL, and 10 U/mL failed to yield a linear response. Thus, as in experiments with GCT, HPCM, and BCM, the CSFs directly stimulated progenitor growth independent of accessory cell populations.

PHA-LCM. In some cases, PHA-LCM supported cell growth with single-hit kinetics, although the progenitor frequency was usually low compared with the frequency obtained by 10% GCT (Fig 4). No significant change in the progenitor frequency was observed after the addition of erythropoietin, nor was there any development of erythroid cultures. In some cases, however, the presence of suppressor or helper effects was evident as deviations from linearity. Thus, the results of progenitor growth with PHA-LCM were unpredictable. In wells containing PHA-LCM, aggregates of cells were often observed, which suggested the presence of residual lectin. Consequently, the effect of PHA alone was investigated.

Effect of PHA and HC on T Cell–Depleted BM Cells

PHA. As shown in Fig 5, PHA-containing medium showed suppressive action on progenitor growth at levels as low as 0.1 μg/mL, whereas at least 5 μg/mL of PHA was required to stimulate ^{3}H-thymidine uptake with lymphocytes (data not shown). In cultures containing higher concentrations of PHA, cells tended to form aggregates. These were distinguishable from myelomonocytic cells colonies by morphology.

HC. HC was found to have an enhancing effect on progenitor growth at concentrations from 10^{-7} to 10^{-6} mol/L (Fig 6). The addition of HC at this concentration increased the progenitor frequency with single-hit kinetics. A concentration of 10^{-4} mol/L always resulted in suppression, and the effect of a 10^{-5} mol/L concentration was subject to variable responses with different donors. Colonies from cultures without HC tended to be smaller and showed signs of early degeneration.

Morphological Evaluation

Evaluation of cells from wells containing colonies by cytochemistry with myeloperoxidase, AS-D-chloroacetate esterase, and nonspecific esterase staining demonstrated a myelomonocytic lineage (data not shown). Colonies from cells cultured with different CSFs were also evaluated morphologically. No significant difference of CSFs on progenitor

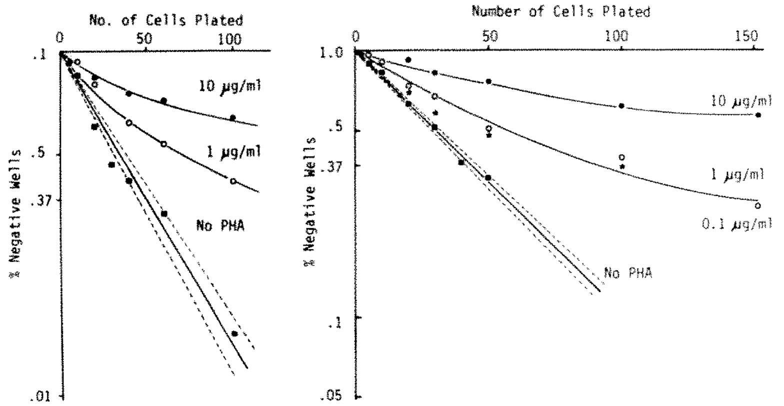

Fig 5. LDA in progenitors with PHA-M plus 10% GCT. A similar suppression was observed in two additional experiments.

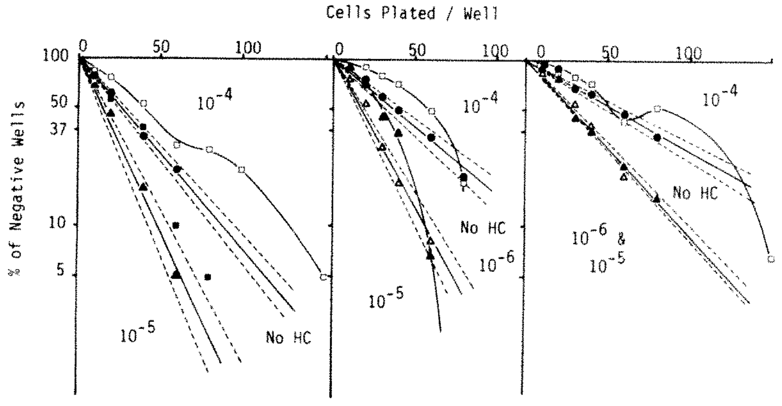

Fig 6. LDA of progenitors with HC. Lower concentrations increased precursor frequency, whereas higher concentrations showed deviations from linearity.

maturation was observed, as shown in Table 3. Erythroid maturation was not observed, even in the presence of erythropoietin.

DISCUSSION

LDA is a quantitative method to determine the frequency of progenitor cells responsive to stimulatory factors in a given population of cells. LDA has proved to be a useful measure for evaluating the factors regulating growth of immunocompetent cells.[21,31-34] To analyze responding GM precursors in a sample that contains mixed cell populations, it is important to exclude the possible effects of other cell types such as monocytes and T lymphocytes on progenitor growth. This can be accomplished because LDA can identify helper or suppressive effects. When the number of cells plated per well is plotted *v* the log of the percent nonresponding wells, linearity indicates that clonal growth of progenitors is unaffected by the cells present and only dependent on the presence or absence of a progenitor. Deviation from linearity indicates the presence of accessory cell populations that have helping or suppressing influences on progenitor growth. In the presence of suppressor cells, plating increasing numbers of cells per well results in an apparent decreasing frequency of progenitors. Hence, we adapted LDA to liquid suspension human GM progenitor cultures to establish an assay for the evaluation of factors regulating hematopoiesis.

Murine hematopoietic progenitors have been assayed in microwell cultures after isolation by fluorescence-activated cell sorting.[35,36] When using an increase in cell number as a response to CSF, accessory cell effects could not be ruled out.[36] LDA of murine BM and spleen GM progenitors without definition of the criteria for clonal growth was hampered by highly variable confidence intervals.[18]

In semisolid culture systems, various criteria have been used to define clonal growth, and the biologic implications of these criteria have not been established. In this study, we have evaluated different criteria for defining clonal growth of progenitors. Most of the criteria used in methylcellulose colony assays (eg, ≥8 cells or ≥40 cells) can also be used in our system. Among them, the criterion ≥10 cells appeared to provide the most consistent results and was used to compare the effect of factors on progenitor growth. We speculate that the larger colonies may represent more primitive progenitors with a greater proliferation potential, whereas the smaller colonies may represent more differentiated cells.

The different responses to CSFs observed in this study suggest that the LDA might measure a different set of progenitors than the semisolid colony assays. In LDA, the best progenitor yield was almost always obtained from cultures containing 10% GCT. BCM and recombinant CSF produced almost the same number of progenitors, but in some instances, PHA-LCM did not. On the contrary, the use of GCT in methylcellulose usually yielded a smaller number of colonies (CFU-GM plus BFU-E plus CFU-GEMM) compared with PHA-LCM (data not shown). For clonal culture of primitive hematopoietic precursors, PHA-LCM was considered to be essential. However, this does not seem to be true in Dexter-type long-term BM culture in which primitive progenitors can be enumerated. Fewer mixed colonies were obtained with 10% PHA-LCM than with 7.5% in semisolid culture.[6,37] Thus, our results indicate that the crude preparation of PHA-LCM cannot be used reproducibly in LDA.

In previous reports, PHA was added to soft agar or methylcellulose cultures to study the effect of activated T cells on progenitor growth. The addition of PHA was thought to promote burst colony formation.[38] In agar culture systems, the addition of PHA alone to T cell–depleted marrow cells was reported to have had no effect on GM progenitor growth at a concentration of 20 μg/mL.[39] Additionally, PHA-stimulated conditioned medium has been used to stimulate the growth of progenitor cells in semisolid and liquid suspension cultures.[40] In LDA, however, a suppressive effect of PHA on progenitor growth was observed at levels as low as 0.1 μg/mL.

HC prevents the growth and function of T cells[41-43] and increases marrow granulocyte production in long-term BM culture.[44] However, reports on the effect of glucocorticosteroids on granulopoiesis in short-term culture systems have been contradictory. Suda et al[45] reported that the addition of HC (10^{-6} mol/L) to T cell– and phagocyte-depleted BM cells increased the number of granulocyte colonies but decreased macrophage colonies in agar culture. Enhanced formation of granulocyte colonies was also observed after the addition of HC in agar[46] and in a diffusion chamber culture system.[47] Slovick et al,[48] however, reported that the addition of 10^{-6} mol/L HC did not significantly change the total number of GM-CFC in agar culture. Cheng et al[43] also noted that the growth of granulocytic colonies in agar was not affected by the presence of up to 10^{-4} mol/L HC. In this study, HC was shown for the first time to have a direct enhancing function on progenitor cell growth in short-term suspension cultures at concentrations of 10^{-7} to 10^{-6} mol/L. Cells cultured with HC also formed larger colonies and tended to be more homogeneous and round, similar to findings reported by Barr et al.[49] We found the use of HC to prevent T cell growth in our assay system to be critical because the possible concomitant growth of T cells may be mistaken for or interfere with GM progenitor growth. The precise action of HC on progenitor cell growth is still unclear; however, LDA provides an approach for further investigation.

Optimum concentrations of CSFs for progenitor growth have been previously reported[50,51] that suggest that promotion of clonal growth by conditioned media is regulated by a balance between stimulators and inhibitors. Thus, the decrease in progenitor frequencies in wells cultured with 1%, 5%, and 20% GCT may be due to a deficiency of CSF at lower concentrations and to the presence of growth-inhibitory substances at higher concentrations. In this respect, LDA has proved to be a reasonable approach for investigation of the mechanism of action of these factors.

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Lymphoma Cell Line (FL-18) and Epstein-Barr Virus–Carrying Cell Line (FL-18-EB) Obtained From a Patient With Follicular Lymphoma: Monoclonal Derivation and Different Properties

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A novel cell line, FL-18, was established from the pleural effusion of a patient with follicular small cleaved cell lymphoma. At the same time, an Epstein-Barr virus (EBV) nuclear antigen (EBNA)-postive cell line, FL-18-EB, was established from the EBV-infected culture of the same pleural effusion cells. Both cell lines had the same monoclonal surface immunoglobulin (IgGκ), and they had the same karyotype as that of the fresh pleural effusion cells in which a reciprocal translocation between the long arm of chromosomes 14 and 18 [t(14;18)(q32;q21)] was detected. Gene rearrangement analysis of immunoglobulin heavy-chain gene (J_H) and κ light-chain gene (Jκ) showed the same rearranged configurations in the two cell lines; however, some morphological and phenotypic differences were found. The FL-18-EB cells, which were morphologically similar to common EBNA-positive lymphoblastoid cell lines of normal B cell origin at the initial phase of culture, were larger than the FL-18 cells and contained multinucleated giant cells. The FL-18 cells lacked cytoplasmic immunoglobulin and were positive for common acute lymphoblastic leukemia antigen (CALLA), whereas the FL-18-EB cells had cytoplasmic immunoglobulin and were negative for CALLA. Thus, the phenotype of FL-18-EB seems to be a result of a shift by EBV infection to a more mature stage in the B cell differentiation pathway than that of FL-18. The paired availability of EBV-free and EBV-infected cell lines of a neoplastic clone is unique and valuable in considering EBV infectibility of neoplastic B cells and resultant phenotypic changes.

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THE TARGET CELL REPERTOIRE of Epstein-Barr virus (EBV) has been studied in terms of EBV nuclear antigen (EBNA) inducibility or the occurrence of transformation by using human neoplastic B cells from diseases such as chronic lymphocytic leukemia,[1-4] prolymphocytic leukemia, and Waldenström's macroglobulinemia.[5] Indeed, a few lymphoid cell lines were considered to carry EBV and definite chromosome aberrations indicative of neoplastic origin,[1,2,5] although some authors insisted that EBV-infected neoplastic cells were not transformed in most cases.[2,6] Further convincing evidence has not been shown regarding the derivation of the alleged EBV-carrying human neoplastic B cell lines.

There is now general agreement that endemic Burkitt's lymphoma cells harbor EBV, whereas nonendemic ones are commonly EBV-free.[7] Two groups have successfully converted EBV-free nonendemic Burkitt's lymphoma cell lines into EBV-carrying ones by EBV infection in vitro.[8,9] Klein et al concluded that EBV infection shifted a nonendemic Burkitt's lymphoma line (Ramos) into a more "activated stage."[10] Notably, however, little is known about EBV infectibility of non-Burkitt's lymphoma cells. We describe here a follicular lymphoma cell line (FL-18) and an EBV-carrying cell line (FL-18-EB) established from the same source. They have the same karyotype and the same pattern of immunoglobulin gene rearrangement; however, they are distinct in terms of morphology and phenotype, probably resulting from the EBV infection itself.

MATERIALS AND METHODS

Case report. A 68-year-old Japanese male noted cervical lymph node swelling in August 1980. Lymph node biopsy in February 1982 revealed follicular small cleaved cell lymphoma.[11] He received combined chemotherapy and achieved complete remission. A pleural effusion developed in July 1984. Cell lines were established from the culture of the pleural effusion cells collected at this time. The patient gave informed consent. He died in November 1984.

Preparation of EBV, infection in vitro, and establishment of the cell lines. Preparation of EBV and infection in vitro were performed as previously reported.[3] Briefly, the culture supernatants of B95-8 cells were centrifuged for two hours at 15,000 g. The pellet was diluted with a nonconcentrated culture supernatant. The pleural effusion cells were washed twice in phosphate-buffered saline and then were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Two weeks later, continuous cell growth was observed, and the established cell line was designated FL-18. At the same time, 10^7 pleural effusion cells were incubated with 0.4 mL of the EBV for 90 minutes at 37°C. The infected cells were washed twice and cultured in the same manner as the untreated culture. They also showed continuous growth and were found to be positive for EBNA 2 months later. This EBNA-positive cell line was designated FL-18-EB. The FL-18 cells were infected in vitro in the same way as the fresh pleural effusion cells. The presence or absence of EBNA-positive cells was tested once a week until 3 months after the infection. The same experiment was repeated three times.

Cytochemical examination, cell surface marker analysis, and EBNA. The pleural, FL-18, and FL-18-EB cells were examined with peroxidase (POX), α-naphthyl acetate esterase (NAE),[12] naphthol AS-D chloroacetate esterase (CAE),[12] and PAS.[13] Spontaneous rosette formation with sheep erythrocytes (E rosette), receptors for the Fc portion of IgG (Fcγ receptor) and receptors for complement (C3b and C3d receptor) were examined as previously described.[14-16] Surface and cytoplasmic immunoglobulin (Ig) was tested by the

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direct immunofluorescence method. Reactivities with monoclonal antibodies were tested by the indirect immunofluorescence method. Monoclonal antibodies used in this study consisted of OKIa-1 for the HLA-DR antigen, B1,[17] B2,[18] J5 for common acute lymphoblastic leukemia antigen (CALLA),[19] Leu-1,[20] PCA-1,[21] EBVCS-4,[22] EBVCS-5,[22] and OKT9.[23] EBNA was tested according to the method of Reedman and Klein.[24]

Cytogenetic study. The pleural effusion cells were processed after short-term culture (24 hours) in RPMI 1640 medium supplemented with 20% FCS at 37°C. The cells were harvested after a 60-minute incubation with Colcemid and then treated with 0.075 mol/L KCl for 20 minutes before they were fixed in methanol acetic acid (3:1). Metaphases were banded by trypsin-Giemsa (G-band).[25] Chromosome analysis of the cultured FL-18 and FL-18-EB cells was done 6 months after the initiation of the culture. Metaphase preparation was performed when the cells showed exponential proliferation. Processing and banding methods were the same as those of the pleural effusion cells.

Gene rearrangement analysis of Ig heavy chain and light chain. Genomic DNA was extracted from the THP-1 (for control), FL-18, and FL-18-EB cells as previously described[26] and digested with *Bam*HI. The digested DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose paper according to the method of Southern.[27] The 3.4-kilobase (kb) *Eco*RI-*Hin*dIII fragment of the joining region of the heavy-chain gene and the 2-kb *Sac*I-*Sac*I fragment of the joining region of the κ chain gene were used as J_H and $J\kappa$ probes. The nick-translated ^{32}P-labeled probes were hybridized to the nitrocellulose filters and visualized on autoradiograms.

Table 1. Cytochemical and Immunologic Markers of Pleural Effusion Cells, FL-18, and FL-18-EB

Markers	Pleural Effusion Cells (%)	FL-18 (%)	FL-18-EB (%)
POX	–	–	–
NAE	–	–	–
CAE	–	–	–
PAS	–	–	+
E rosette	<1	0	0
Fcγ receptor	17	4	1
C3b receptor	<1	3	1
C3d receptor	NT	2	18
Surface Ig			
κ	1	75	15
λ	0	0	0
μ	0	0	0
γ	0	75	14
α	0	0	0
σ	0	0	0
Cytoplasmic Ig	0	0	κ, 32; γ, 28
HLA-DR	80	99	70
B1	87	99	77
B2	NT	8	49
CALLA	1	67	0
Leu-1	10	0	0
PCA-1	NT	0	0
OKT9	NT	53	63
EBVCS-4	NT	0	73
EBVCS-5	NT	0	63
EBNA	0	0	100

Abbreviation: NT, not tested.

RESULTS

Morphological findings. Cytocentrifuged smears of the FL-18 and FL-18-EB cells are shown in Fig 1. The pleural effusion cells were small cleaved lymphoid cells that were morphologically the same as the lymph node cells with a diagnosis of follicular lymphoma. The FL-18 cells were also small to medium in size. The FL-18-EB cells, however, were larger than the FL-18 cells and contained multinucleated giant cells. The FL-18-EB cells were indistinguishable from the cells of common lymphoblastoid cell line of normal B cell origin at the early phase of the culture because the FL-18-EB cells formed firm clumps and exhibited pseudopods.[28]

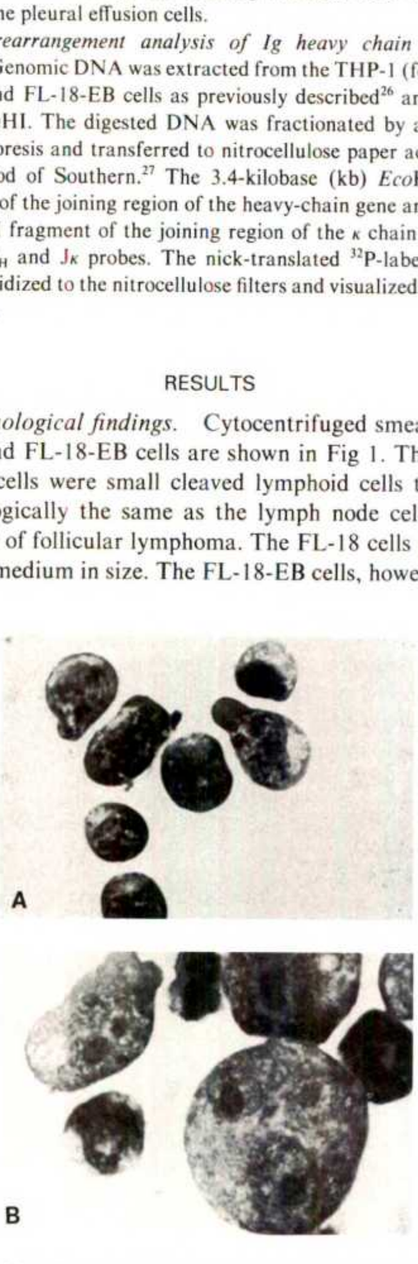

Fig 1. May-Giemsa stain of cytocentrifuged smears of (A) FL-18 and (B) FL-18-EB.

Cytochemical examination, surface marker analysis, and EBNA. Table 1 shows the results of cytochemistry and cell surface phenotype including reactivities with the monoclonal antibodies and EBNA. The pleural, FL-18, and FL-18-EB cells were all negative for POX, NAE, or CAE. The FL-18-EB cells were positive for PAS, although the pleural effusion cells and FL-18 cells were negative. The fresh pleural effusion cells were positive for HLA-DR and B1 and were negative for E rosette or Leu-1, whereas surface Ig was not detected. B2 was not tested in the pleural effusion cells. The FL-18 cells were also positive for HLA-DR and B1. Unlike the pleural effusion cells, the FL-18 cells had surface Ig (IgGκ) and were positive for CALLA. They expressed the B2 antigen only to a limited degree. The pleural effusion cells and the FL-18 cells were negative for EBNA. Virtually all of the cells of FL-18-EB were positive for EBNA. They were also positive for HLA-DR and B1 and had monoclonal surface Ig (IgGκ). They had cytoplasmic Ig and expressed the B2 antigen in a major population. They were negative for CALLA. Neither of the two cell lines was reactive with PCA-1. EBVCS-4 and -5, which have been reported to react specifically with common lymphoblastoid cell lines of B cell origin, reacted with FL-18-EB but not with FL-18.

Cytogenetic study. Table 2 shows the results of the chromosome analysis of the fresh pleural, FL-18, and FL-

Table 2. Summary of the Karyotype of the Pleural Effusion Cells, FL-18, and FL-18-EB

Pleural effusion cells	49, XY, +X, +7, +12, dir ins(13;8) (q14;q22q24), t(8;22) (q24;q13), t(14;18) (q32;21)
FL-18	49, XY, +7, +12, −17, +del(X) (p11), del(6) (p11), dir ins(13;8) (q14;q22q24), t(8;22) (q24;q13), t(14;18) (q32;q21), +der(17)t(17;?) (q23;?)
FL-18-EB	49, XY, +X, +7, +12, dir ins(13;8) (q14;q22q24), t(8;22) (q24;q13), t(14;18) (q32;q21) (Tetraploid) 94, XX, −Y, 4n±{+7, +7, +12, +12}, 2x dir ins(13;8) (q14;q22q24), 2x t(8;22) (q24;q13), 2x t(14;18) (q32;q21)

18-EB cells. The fresh lymphoma cells were hyperdiploid, and the modal chromosome number was 49 with gains of chromosomes 7, 12, and an X. They showed a reciprocal translocation between the long arms of chromosomes 14 and 18 [t(14;18)(q32;q21)]. In addition, complex chromosome rearrangements involving nos. 8, 13, and 22 were observed. FL-18 also had a 14q+ marker chromosome that was the result of a translocation between nos. 14 and 18. The modal karyotype was the same as that of the pleural effusion cells. In some cells, however, additional abnormal chromosomes such as 6p−, 17q+, and Xp− were found. The modal karyotype of FL-18-EB was the same as that of the fresh lymphoma cells. The additional abnormal chromosomes observed in FL-18 were not found in FL-18-EB. Fifty percent of the karyotypes of FL-18-EB showed a tetraploid genome. None of the FL-18-EB cells showed a normal karyotype.

Gene rearrangement analysis of Ig heavy chain and light chain. Fig 2 shows the gene rearrangement analysis of Ig heavy chain and κ chain when using *Bam*HI as a restriction endonuclease. Two clonally rearranged bands in the analysis using J_H as a probe and one rearranged band in that using $J\kappa$ were observed at the same positions in FL-18 and FL-18-EB. The pleural effusion cells were not available for this study.

In vitro infection of FL-18 with EBV. Virtually no EBNA-positive cells were induced among the treated FL-18 cells.

DISCUSSION

FL-18 is thought to be derived from the lymphoma cells in the pleural effusion of a patient with follicular small cleaved cell lymphoma because it carried the same karyotype as that of the pleural effusion cells and was negative for EBNA. Cytogenetically, FL-18 had a reciprocal translocation between the long arms of chromosomes 14 and 18 [t(14;18)(q32;q21)], which is known to be specific for follicular lymphoma.[29] The details of the karyotype of FL-18 have been reported previously.[30] FL-18 had monoclonal surface Ig globulin (IgGκ), although no surface or cytoplasmic Ig was detected in the pleural effusion cells.

We also established an EBV-carrying cell line, FL-18-EB. It is unlikely that FL-18-EB is a common lymphoblastoid cell line derived from normal B cell in the pleural effusion because FL-18-EB had the same karyotype and monoclonal Ig as FL-18. Moreover, Ig gene rearrangement analysis

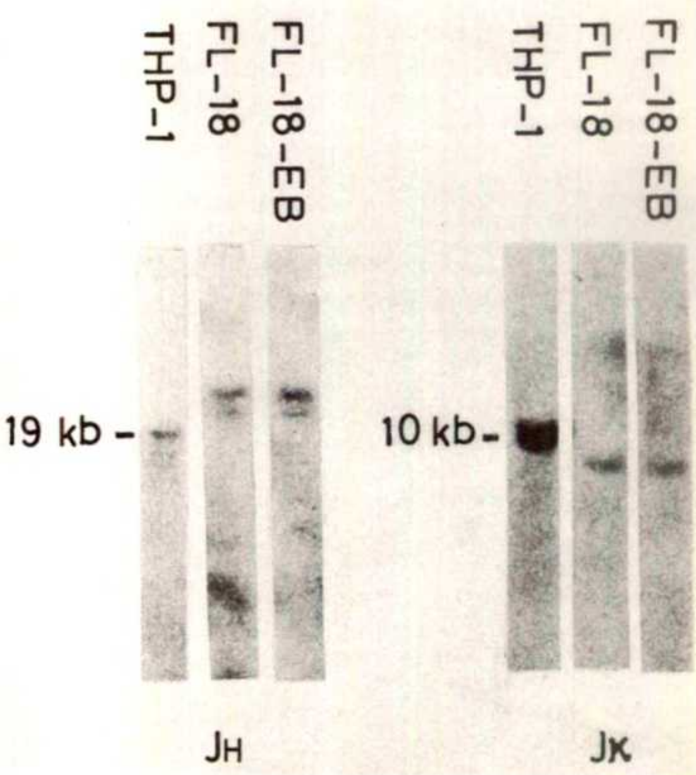

Fig 2. Southern blot analysis of DNA from THP-1 (for control), FL-18, and FL-18-EB when using J_H and $J\kappa$ as probes. The germlines of 19 kb and 10 kb are indicated respectively. Rearranged bands are demonstrated at the same positions in FL-18 and FL-18-EB.

using J_H and J_κ as probes revealed the same rearranged bands in the two cell lines, thereby indicating their clonal identify.

Several morphological and phenotypic differences were found between FL-18 and FL-18-EB. Unlike the FL-18 cells, the FL-18-EB cells were larger and contained multinucleated giant cells. At the early stage of passage, the FL-18-EB cells were morphologically indistinguishable from common lymphoblastoid cell lines of normal B cell origin. Some investigators have described similar changes accompanying EBV transformation of neoplastic B cells. Karande et al reported that EBV-transformed chronic lymphocytic leukemia cells were morphologically similar to common lymphoblastoid cell lines.[1] Fresen and zur Hausen also reported that their EBV-infected BJAB cells contained multinucleated gaint cells.[9] In addition, the expression of the EBVCS-4– or -5–determined antigen can be regarded as a feature shared by common lymphoblastoid cell lines with FL-18-EB but not with FL-18. It should be noted that the antigen is not related specifically to EBV infection of the cell lines because Raji cells do not express this antigen.

The FL-18-EB cells expressed the B2 antigen, whereas the FL-18 cells hardly did. The C3d receptor detected with B2 is thought to be a receptor molecule for EBV.[31-33] The augumentation of the C3d receptor on the FL-18-EB cells may be a result of EBV infection as has been described.[10] Alternatively, EBV may have infected the C3d receptor–positive subpopulation of the fresh lymphoma cells, thereby leading to the generation of FL-18-EB, although the extent to which the fresh cells expressed the B2 antigen is unknown. The FL-18 cells lacked cytoplasmic Ig and were positive for CALLA, whereas the FL-18-EB cells had cytoplasmic Ig and did not express CALLA. It is generally thought that, in the differentiation from B cells to plasma cells, cytoplasmic Ig appears and surface Ig fades away.[34] CALLA, which is expressed on most of the precursors of B cells such as common ALL and a part of B cells, disappears in the differentiation of B cells.[34] Therefore, FL-18-EB seems to be closer to plasma cells than FL-18 is, although the FL-18-EB cells did not react with PCA-1, which is thought to be specific for plasma cells.[21] These phenotypic differences are probably due to EBV infection itself rather than to the clonal difference of the two cell lines. To confirm the effect of EBV infection on the lymphoma cells, we tried to infect the FL-18 cells with EBV in vitro. No EBNA-positive cells were induced in any experiments. However, further studies are necessary to prove the absence of the infectibility.

The FL-18 and FL-18-EB cell lines may be of value for examining two isogenic cell lines at slightly different stages of maturation.

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Identification and Characterization of a Unique Subpopulation (CALLA/CD10-Negative) of Human Neutrophils Manifesting a Heightened Chemotactic Response to Activated Complement

By Robert T. McCormack, Robert D. Nelson, Dennis E. Chenoweth, and Tucker W. LeBien

We have previously demonstrated that human neutrophils synthesize the common acute lymphoblastic leukemia antigen (CALLA/CD10). To determine whether CALLA/CD10-positive and -negative neutrophils have similar or distinct functional attributes, we sorted normal peripheral blood neutrophils for CALLA/CD10 expression and compared their chemotactic ability. Surprisingly, the low-frequency (~5%), CALLA/CD10-negative neutrophils displayed a dramatically heightened chemotactic response to activated complement (C′) that was (*a*) specific for C′, (*b*) not observed with other minor subpopulations of neutrophils, (*c*) not due to previous activation in vivo or in vitro, and (*d*) apparently not due to an increase in C5a receptors. These results underscore the concept of neutrophil heterogeneity and prompt the hypothesis that CALLA/CD10-negative neutrophils may participate in an inflammatory response to trauma involving complement activation.

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THE PHENOTYPIC AND FUNCTIONAL heterogeneity of peripheral blood mononuclear cells has been largely defined by using monoclonal antibodies.[1,2] Functional heterogeneity of human polymorphonucleated neutrophils was intially suggested over 60 years ago,[3] and more recent studies have documented heterogeneity of neutrophil function,[4-13] density,[14,15] and RNA/protein biosynthesis.[16] Monoclonal antibodies have also been applied in further attempts to unravel heterogeneity of neutrophil origin and function.[17-24]

The common acute lymphoblastic leukemia antigen (CALLA/CD10) is a 95-kD cell surface molecule originally characterized on ALL cells by Greaves et al.[25] CALLA/CD10 has subsequently been identified on normal and malignant cells of both hematopoietic and nonhematopoietic origin,[26-34] including neutrophils.[35,36] We have recently confirmed and extended the observations of others[35,36] by showing that neutrophils synthesize and insert CALLA/CD10 into their membranes.[37] However, blocking studies with the anti-CALLA/CD10 monoclonal antibody BA-3 failed to ascribe any role for CALLA/CD10 in neutrophil function.[37]

In the experiments reported herein, we have identified a rare subpopulation (~5%) of segmented peripheral blood neutrophils from normal donors characterized by (*a*) an absence of cell surface CALLA/CD10 and (*b*) the presence of a heightened chemotactic responsiveness to activated complement (C′). The potential biologic significance of our results is discussed.

MATERIALS AND METHODS

Monoclonal antibodies. The anti-CALLA/CD10 monoclonal antibody BA-3 is an IgG2b antibody that was produced and characterized in this laboratory.[26] AHN-1 is an IgM monoclonal antibody that recognizes a 150/105-kD complex on neutrophils[38] and was kindly provided by Dr Keith Skubitz, University of Minnesota. OkM-1, a monoclonal antibody that recognizes the C3bi receptor,[39] was purchased as a fluorescein isothiocyanate conjugate (FITC-MO-1) from Coulter Diagnostics, Hialeah, FL.

Purification of neutrophils. Peripheral blood neutrophils were purified from heparinized whole blood by using Ficoll-Hypaque centrifugation followed by hypotonic lysis of erythrocytes. Final preparations contained >95% neutrophils, with the remainder of the cells being contaminating eosinophils and basophils.

Immunofluorescence analysis and cell sorting. Neutrophils were stained with monoclonal antibodies and analyzed by indirect immunofluorescence as previously described.[37] Cell sorting of peripheral blood neutrophils was accomplished by using a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Mountain View, CA) equipped with a 5-W argon ion laser. The distribution of CALLA/CD10-positive and -negative neutrophils was determined on each donor, and the fluorescent gains were adjusted to collect positive and negative cells. At a sort rate of 3 to 4 × 10^3 cells/s, we could collect enough CALLA/CD10-negative neutrophils for functional studies in four hours. When the percentage of CALLA/CD10-negative peripheral blood neutrophils was less than 5% of the total neutrophils stained, the gains were increased to 5% of the total cells to facilitate the sorting time. CALLA/CD10-positive and -negative neutrophils were collected into 15-mL conical tubes containing phosphate-buffered saline (pH 7.2) with 2.5% fetal bovine serum and 0.02% sodium azide (PBS-F). The tubes were shaken periodically to keep the neutrophils in suspension. After sorting, the cells were washed twice in PBS-F, counted, and assayed for chemotactic function. A sample from each CALLA/CD10-positive and -negative fraction was removed and examined for cell morphology by using Wright-Giemsa staining. Four populations of cells from each donor were examined for chemotaxis: (*a*) unstained, unsorted cells that served as a control for the effects of sorting on chemotaxis; (*b*) cells stained with BA-3 and FITC–goat antimouse (GAM) but not sorted to control for the effects of antibody binding on chemotaxis; (*c*) cells stained and sorted as CALLA/CD10-positive; and (*d*) cells stained and sorted as CALLA/CD10-negative. Unstained, unsorted and stained, unsorted cells were held on ice during the sort to minimize lability.

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The aforementioned procedure was also followed for sorting AHN-1–positive and –negative peripheral blood neutrophils with the following exception: only two populations of cells were prepared for each donor, unstained/unsorted cells and AHN-1–negative cells (see Results).

Unsorted, purified neutrophils were subjected to two-color flow cytometric analysis to determine the relationship between expression of CALLA/CD10 and C5a receptors. Neutrophils at 1×10^6/mL were first stained with FITC-C5a for 25 minutes at room temperature at a final C5a concentration of 5 or 50 nmol/L.[40] The cells were washed once with PBS-F and sequentially stained with BA-3 or control ascitic fluid (both 1:100), GAM-biotin (Boeringer Mannheim Biochemicals, Indianapolis) at 7.5 µg/mL, and 5 µL stock phycoerythrin-avidin (Becton Dickinson), all for 30 minutes at 4°C in a final volume of 100 µL interspersed with two washes in PBS-F. Two-color analysis was conducted with a FACS 440 analyzer (Becton Dickinson) with a 488-nm excitation wavelength from an argon laser. FITC and phycoerythrin emission wavelengths were detected by using 535-20 and 575-15 dichroic interference filters, respectively. Phycoerythrin emission detection was corrected for stray FITC emission, and the results were analyzed on a Consort 40 computer (Becton Dickinson) using a DISP2D program.

To examine the relationship between CALLA/CD10 and C3bi receptor expression, neutrophils were sorted into CALLA/CD10-positive and -negative fractions by using BA-3, GAM-biotin and phycoerythrin-avidin. Sorted cells were washed twice with PBS-F, and CALLA/CD10-positive and -negative and unsorted cells were then stained with FITC–MO-1 according to the manufacturer's instructions. The cells were then examined for green fluorescence.

Chemotaxis. Chemotaxis was performed by the agarose method of Nelson et al[41] with gelatin as a protein source. Serum collected from the same donor whose neutrophils were being assayed served as an eventual source of C′. *N*-Formyl-methionyl-leucyl-phenylalanine (F-mlp, Pierce Biochemicals, Rockford, IL) was used at a final concentration of 5×10^{-5} mg/mL.

Statistics. The two-tailed Student's test was used as a test for statistical significance.

RESULTS

Chemotaxis and random migration of CALLA/CD10-positive and CALLA/CD10-negative peripheral blood neutrophils. We[37] and others[35,36] have previously reported that ~95% of normal peripheral blood neutrophils express CALLA/CD10. To determine the relationship between CALLA/CD10 expression and neutrophil function, we sorted peripheral blood neutrophils from six normal donors into CALLA/CD10-positive and -negative populations and tested for chemotactic response to C′ and F-mlp. The results in Table 1 show that there was no significant difference in the chemotactic response to C′ between unstained/unsorted, stained/unsorted, and sorted CALLA/CD10-positive cells. The slight inhibition observed in the stained/unsorted and sorted CALLA/CD10-positive cells is consistent with other data from this laboratory indicating that BA-3 has a slight inhibitory effect on chemotaxis.[37] In striking contrast, the chemotactic response of CALLA/CD10-negative cells toward C′ (5.52 ± 0.99) was significantly greater than that of either CALLA/CD10-positive cells (3.19 ± 1.48, $P < .001$) or unstained/unsorted cells (3.68 ± 1.51, $P < .001$). Differences between CALLA/CD10-negative neutrophils and unstained/unsorted neutrophils are most meaningful because the latter population represents the true migratory capacity of neutrophils in vitro. CALLA/CD10-negative neutrophils from male and female donors were equally responsive to C′ (data not shown). The heightened chemotactic response of CALLA/CD10-negative cells was only observed for C′. There were no significant differences in the chemotactic response to F-mlp or in random migration among the cell populations tested. The similarity in chemotaxis to C′ of unstained/unsorted, stained/unsorted, and CALLA/CD10-positive cells in addition to the absence of differences in chemotaxis to F-mlp of all populations tested indicates that the cell sorting procedure was not unduly detrimental to chemotaxis.

Table 1. Chemotactic Response of CALLA/CD10-Positive and CALLA/CD10-Negative Peripheral Blood Neutrophils

Cells		Stimulus		
Stained	Sorted	C′	F-mlp	RM
–	–	3.68 ± 1.51[*]	3.93 ± 0.80	1.67 ± 0.36
+	–	3.22 ± 1.72	3.70 ± 1.92	1.62 ± 0.37
+	CALLA/CD10+	3.19 ± 1.48	3.98 ± 0.80	1.61 ± 0.38
+	CALLA/CD10−	5.52 ± 0.99	4.10 ± 0.74	1.93 ± 0.52

Neutrophils were separated into CALLA/CD10-positive or -negative fractions by using a FACS IV and tested for chemotaxis. CALLA/CD10-negative cells were assayed in duplicate or triplicate for each chemoattractant, depending upon cell yield. All other populations were assayed in triplicate.

Abbreviation: RM, random migration.

[*] Means ± SD of the chemotactic response (measured in centimeters) from six donors (three males, three females).

Figure 1 is a composite of micrographs showing CALLA/CD10-positive (Fig 1A, C) and CALLA/CD10-negative (Fig 1B, D) neutrophils migrating in response to C′ in the agarose assay. These micrographs provide direct evidence that the predominant CALLA/CD10-negative cells exhibiting a heightened chemotactic response to C′ were segmented neutrophils that were morphologically indistinguishable from CALLA/CD10-positive neutrophils. Furthermore, there were no CALLA/CD10-positive neutrophils contaminating the CALLA/CD10-negative population when checked by immunofluorescence microscopy. Contaminating CALLA/CD10-negative eosinophils and basophils constituted less than 6% of the CALLA/CD10-negative cell population and did not contribute to the leading migratory front. CALLA/CD10-positive cells were composed entirely of mature neutrophils. There were no consistent differences observed in the number of cells constituting the leading front (as shown in the micrograph) in the CALLA/CD10-positive or -negative populations.

Specificity of the heightened chemotactic response. To determine whether the heightened chemotactic response to C′ was unique to CALLA/CD10-negative neutrophils, neutrophils were sorted according to reactivity with the monoclonal antibody AHN-1. AHN-1 is a neutrophil-specific antibody recognizing an antigen that, like CALLA/CD10, is expressed on 95% of peripheral blood neutrophils. Because AHN-1 has been shown to inhibit neutrophil chemotaxis,[42] only two populations of neutrophils were tested: unstained/unsorted and AHN-1–negative. Results in Table 2 show that, unlike CALLA/CD10-negative neutrophils, AHN-

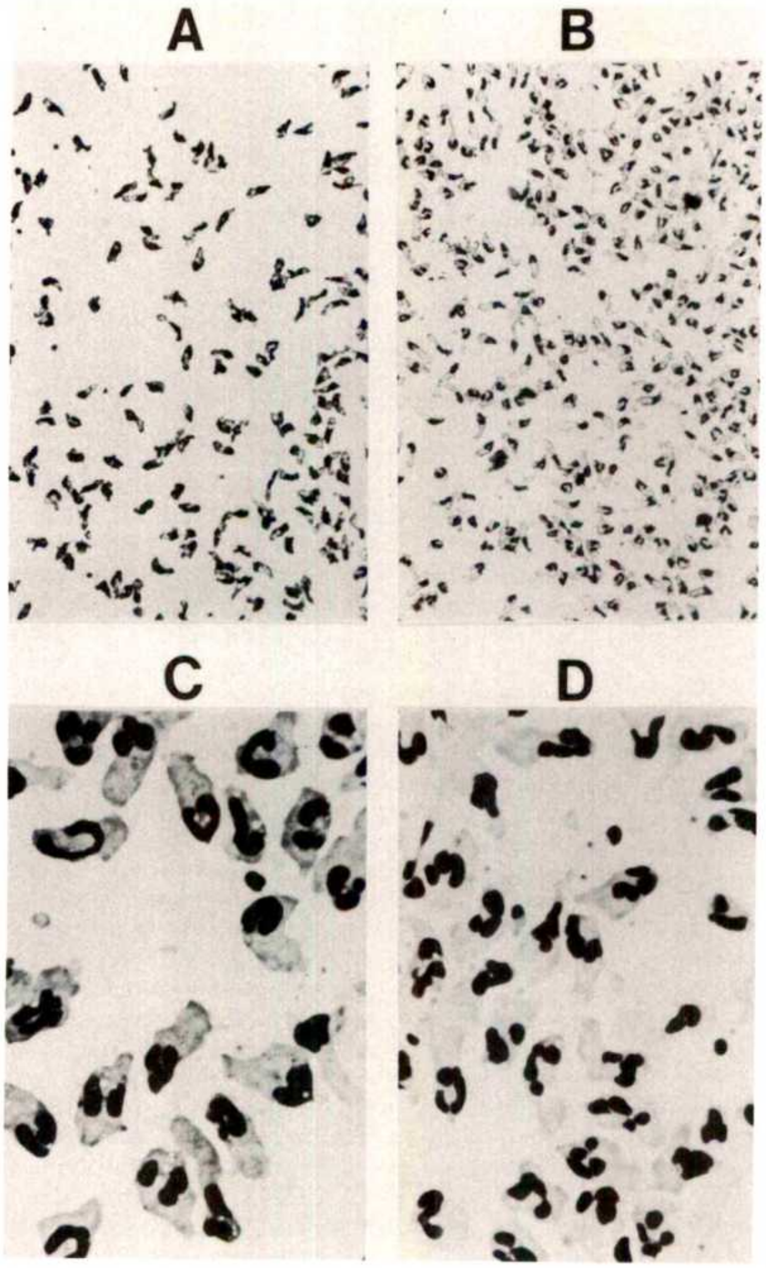

Fig 1. Light micrographs of CALLA/CD10-positive (A, C) and negative (B, D) neutrophils migrating in response to C′ in the agarose assay. (Panels A and B, original magnification × 10; panels C and D original magnification × 100 of cells at the leading migratory front; the chemoattractant is to the left in all photographs.

1–negative neutrophils did not exhibit a heightened chemotactic response to C′ (2.02 ± 0.05) when compared with unstained/unsorted neutrophils (2.72 ± 0.48). Indeed, AHN-1–negative neutrophils were less responsive to both C′ and F-mlp.

Relationship between CALLA/CD10 and C5a receptor expression on peripheral blood neutrophils. To determine whether the heightened chemotactic response to C′ was due to increased C5a receptors on CALLA/CD10-negative cells, we performed two-color flow cytometric analysis on purified neutrophils by using FITC-C5a and BA-3 plus GAM-biotin and phycoerythrin-avidin as described in Materials and Methods. Figure 2 is a representative contour plot from one of three normal donors tested by using 50 nmol/L FITC-C5a. The vast majority of neutrophils (95%) coexpressed CALLA/CD10 and C5a receptors as shown in the upper right quadrant. CALLA/CD10-negative, C5a receptor–positive and CALLA/CD10-negative, C5a receptor–negative cells each represented 2% to 3% of the total cells analyzed as shown in the lower right and lower left quadrants, respectively. It is apparent that no distinct population of CALLA/CD10-negative neutrophils express C5a receptor levels greater than CALLA/CD10-positive neutrophils, although there is some heterogeneity in C5a receptor levels in CALLA/CD10-negative neutrophils. Similar results were obtained from two other donors when using 5 and 50 nmol/L FITC-C5a. We conclude that the heightened chemotactic response of CALLA/CD10-negative neutrophils to C′ is not attributable to increased numbers of C5a receptors on CALLA/CD10-negative neutrophils.

Table 2. Chemotactic Response of AHN-1-Positive and AHN-1-Negative Peripheral Blood Neutrophils

Cells		Stimulus		
Stained	Sorted	C′	F-mlp	RM
–	–	2.72 ± 0.48*	3.43 ± 0.32	1.19 ± 0.10
+	AHN-1 –	2.02 ± 0.51	2.67 ± 0.25	1.02 ± 0.04

Neutrophils were separated into AHN-1-positive or -negative fractions by using a FACS IV, and the AHN-1-negative cells were tested for chemotaxis.

*Means ± SD of the chemotactic response (measured in centimeters) from three donors.

Relationship between CALLA/CD10 and C3bi receptor expression on peripheral blood neutrophils. One possible explanation for the increased chemotactic response displayed by CALLA/CD10-negative neutrophils is that these cells were preactivated, either in vivo or in vitro (as a consequence of sorting). We therefore sorted neutrophils into CALLA/CD10-positive and -negative populations and examined each for C3bi receptor expression, a receptor previously shown to dramatically increase after neutrophil activation.[43] Ninety-eight percent of sorted, CALLA/CD10-positive neutrophils were C3bi receptor-positive with a mean channel of 620. Eighty-seven percent of sorted, CALLA/CD10-negative neutrophils were C3bi receptor-positive with a mean channel of 607. Unstained, unsorted neutrophils from the same donor were 89% C3bi receptor-positive with a mean channel of 619. These results confirmed data obtained from three donors when using two-color analysis to compare CALLA/CD10 and C3bi receptor expression (data not shown). Taken collectively, these results indicate that the heightened chemotactic response of CALLA/CD10-negative neutrophils to C′ is not due to significant selective activation before chemotactic analysis.

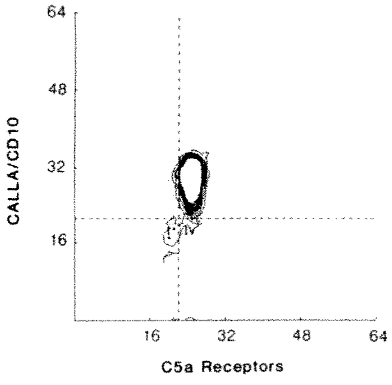

Fig 2. Two-color flow cytometric contour plot of human peripheral blood neutrophils analyzed for expression of CALLA/CD10 (y-axis) and C5a receptors (x-axis). Staining of neutrophils was conducted as described in Materials and Methods with 50 nmol/L FITC-C5a. Based on background staining the contour plot was divided into quadrants representing CALLA/CD10-positive/C5a receptor-negative neutrophils (upper left quadrant), CALLA/CD10-positive/C5a receptor-positive neutrophils (upper right quadrant), CALLA/CD10-negative/C5a receptor-negative neutrophils (lower left quadrant), and CALLA/CD10-negative/C5a receptor-positive neutrophils (lower right quadrant).

DISCUSSION

CALLA/CD10 is a 95 kD cell surface molecule originally identified on leukemic cells from patients with acute lymphoblastic leukemia[25] and subsequently shown to be expressed on human neutrophils.[35,36] We recently demonstrated that neutrophils actively synthesize CALLA/CD10 and also observed that leukemic cell and neutrophil CALLA/CD10 have similar if not identical protein backbones.[37] We[37] and others[36] have shown that myelopoietic CALLA/CD10 expression occurs at the level of the segmented neutrophil, 90% to 95% of segmented neutrophils being CALLA/CD10-positive. Because only 5% to 10% of segmented neutrophils are CALLA/CD10-negative, we hypothesized that these cells were possible precursors of CALLA/CD10-positive neutrophils and may be functionally immature. The experiments in Table 1 were initiated to test this hypothesis and showed, quite surprisingly, that the low-frequency, CALLA/CD10-negative neutrophils manifest a heightened chemotactic response to C′. Additionally, the isolation and analysis of a population of peripheral blood neutrophils that lack another cell surface antigen (AHN-1) did not result in a similar increased chemotactic response to C′ (Table 2). Collectively, these results document the existence of a low-frequency subpopulation of neutrophils manifesting an increase in chemotaxis toward C′ that is not demonstrable with other chemoattractants (eg, F-mlp) or minor neutrophil subpopulations.

Several explanations for the heterogeneous chemotactic responses of neutrophils have been suggested, including differences in ligand binding and cell age. The subject of ligand binding to individual cells and its relation to chemotaxis has been controversial. Harvath and Leonard[13] divided peripheral blood neutrophils into migrating and nonmigrating populations but showed that both populations bind F-mlp equally. Conversely, Seligmann et al[11] have shown that neutrophils bind fluoresceinated F-mlp in a heterogeneous manner, which offers a possible explanation for the heterogeneous chemotactic response of individual neutrophils to this ligand. Using two-color flow cytometric analysis we found no CALLA/CD10-negative neutrophil population with C5a receptor levels clearly above CALLA/CD10-positive neutrophils. These results agree with those of Van Epps and Chenoweth who found no identifiable neutrophil subpopulations with greatly increased numbers of C5a receptors.[40] Our results imply that the heightened chemotactic response to C′ of CALLA/CD10-negative cells is best explained by a postligand binding event, but we cannot exclude the possibility of subtle differences in receptor affinity between CALLA/CD10-positive and -negative neutrophils.

The concept of neutrophil heterogeneity has been reviewed,[44] but its biologic significance remains uncertain. It seems likely that the phenotypic/functional differences detected in this study and others[17-23] reflect differences in gene regulation and expression between subpopulations. Unfortunately, little is known concerning the regulation of C5a receptor expression and the signal transduction that follows binding of C5a. Because the difference in responsiveness is quantitative rather than qualitative, we must assume that CALLA/CD10-negative and -positive neutrophils are equally endowed with the necessary machinery (ie, receptors and second messengers) to manifest an effective response. However, preliminary evidence indicates that although distinct subpopulations are not identifiable by quantification of C5a receptors,[40] not all C5a receptors are associated with the cytoskelton (D.E. Chenoweth, unpublished observations). The relationship between cytoskeleton-associated/nonassociated C5a receptors and CALLA/CD10 expression requires further investigation.

The relationship between the heightened chemotactic response to C′ and the absence of surface CALLA/CD10 is unclear. The presence or absence of CALLA/CD10 may be fortuitous, or the acquisition of CALLA/CD10 may be functionally significant. The possible role of CALLA/CD10-negative neutrophils in an inflammatory response in vivo should be pursued.

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Peroxidase-Containing Microgranules in Human Neutrophils: Physical, Morphological, Cytochemical, and Secretory Properties

By R.T. Parmley, W.G. Rice, J.M. Kinkade Jr, C. Gilbert, and J.C. Barton

A microgranule fraction, isolated from human neutrophils by using a novel high-resolution Percoll density gradient system contained granules with the lowest density and diameter when compared with 12 other isopycnic granule fractions. Ultrastructurally, from 34% to 50% of the microgranules showed homogeneous diaminobenzidine (DAB) staining under conditions for localizing peroxidase reactivity. The presence of myeloperoxidase (MPO) was further confirmed by biochemical and spectral analysis and immunodiffusion methods. Periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) intensely stained vicinal glycols in the matrix of greater than 97% microgranules in contrast to the weak or absent staining seen in larger primary granules. Directly sampled segmented neutrophils contained small DAB- and PA-TCH-SP-positive granules, which often appeared in clusters. These DAB-positive microgranules selectively remained within the cells after stimulation of exocytosis with the calcium ionophore A23187. The enriched DAB-positive microgranule fraction recovered from A23187-treated cells also contained lysozyme and β-glucuronidase but lacked vitamin B_{12} binding protein activity. A similar small, DAB- and PA-TCH-SP-positive granule type was also identified in normal promyelocytes and was the predominant or only granule type observed in leukemic or preleukemic myeloid cells from four patients. This study demonstrates a unique subpopulation of MPO-containing microgranules in normal and leukemic human myeloid cells that are distinguished from (other) primary granules by their extremely low density, small size, content of complex carbohydrates, and resistance to secretion.

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PREVIOUS ULTRASTRUCTURAL[1-5] and biochemical studies[6-8] of human neutrophils have identified two major types of cytoplasmic granules, large myeloperoxidase (MPO)-containing primary granules and smaller secondary granules lacking peroxidase activity. However, a number of recent studies[9,10] indicate considerably more heterogeneity among the granules. Rice et al,[9] using a high-resolution Percoll density gradient system, isolated and characterized 13 granule fractions. Among the five higher-density MPO-containing granule fractions, a unique, large granule enriched in microbicidal defensin polypeptides was observed.[11] At least three different forms of MPO have been identified biochemically in human neutrophils.[12,13] These forms show differences in subunit structure and detergent extractability, which indicates differences in compartmentalization or association with granule membranes.[13] In addition, these different forms of MPO appear to be under separate secretory control and are differentially distributed among isolated granules of differing density.[14,15] Taken together, these results indicate the existence of several physically and functionally distinct MPO-containing neutrophil granules rather than a single MPO-containing granule previously termed a primary or azurophil granule.

A variety of apparently distinct small granule populations containing sulfated glycosaminoglycans, acid hydrolases,[16,17] gelatinase,[18] alkaline, phosphatase,[19] elastase,[20] and catalase[21] have been reported in biochemical and/or ultrastructural studies. Ackerman has previously observed small granule profiles (0.1 to 0.25 µm) in early promyelocytes[1] and clusters of small diaminobenzidine tetrahydrochloride (DAB)-reactive granules in metamyelocytes and segmented neutrophils[2]; however, little attention has been directed to these granules since their original description. Recently, these granules have been further characterized with the observation of microtrabeculae connecting the granules when observed by stereo high-voltage electron microscopy.[22] The present study reexamines the microgranule population in neutrophils and demonstrates that their DAB staining corresponds to the presence of MPO and that the granules differ dramatically from typical primary granules in size, density, carbohydrate cytochemistry, and response to secretory stimuli. The granules were identified in isolated neutrophil granule preparations as well as in intact neutrophils, leukemic myeloid cells, and ionophore-treated neutrophils. A preliminary report of this observation has been previously published.[23]

MATERIALS AND METHODS

Separation of granules. Concentrated samples of human neutrophils were obtained from healthy adults undergoing continuous-flow leukapheresis. Neutrophils were further purified to $>97\%$ purity after two cycles of hypotonic lysis of residual erythrocytes[24] and subsequent centrifugation through lymphocyte separation medium[25] to remove monocytes and lymphocytes. Purified cells were lysed, the granule-rich postnuclear supernatant (PNS) was prepared, and the high- (H) and low (L)-density Percoll gradients were performed as described previously by Rice et al.[9] The H and L gradients consisted of fused multiple-step preformed layers of different densities. These gradients were designed to maximize resolution of 13 isopycnic granule fractions that had been initially identified by using continuous, self-generated Percoll density gradients. The gradients were fractionated, and appropriate fractions were pooled and centrifuged for three hours at 100,000 g at 4°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc, Palo Alto, CA). The granule pellet was collected, washed with phosphate buffered saline (PBS) and recentrifuged at 27,000 g for 15 minutes at 4°C in a Dupont/Sorvall (Willmington, DE) SM-24 rotor to obtain a purified granule pellet.

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Assay of granule components. Granule pellets that were to be assayed for MPO were suspended in 0.3% cetyltrimethylammonium bromide in PBS, pH 7.4, and exposed to five cycles of freezing and thawing. The granule lysate was spectrophotometrically assayed for MPO activity by using guaiacol as the electron donor,[14] and an increase in 1 absorbance unit at 470 nm in one minute was defined as 1 unit of activity. Protein concentrations were measured according to Lowry et al.[26]

MPO protein was quantitated by spectroscopy. Difference spectra from 400 to 600 nm were measured in dithionite-reduced minus oxidized samples by using an Aminco DW-2a spectrophotometer (American Instrument Co, Silver Spring, MD). The samples were homogenized in 0.1% Triton X-100, and an absorbance coefficient of 75/mmol/L/cm was used to quantitate the characteristic MPO peak at 472 nm.[27] MPO in microgranules was also identified immunologically by using Ouchterlony double-immunodiffusion[28] plates and a monospecific rabbit antibody prepared against purified normal human neutrophil MPO as previously described.[12]

Activity of β-glucuronidase was measured as described elsewhere,[14] and vitamin B_{12} binding proteins were measured by the rapid charcoal assay described by Kane et al.[29] Amersham, Arlington Heights, IL, supplied the ^{57}Co-vitamin B_{12} (specific activity, 10 to 20 μCi/μg). Lysozyme activity was determined by using the lysoplate method of Osserman and Lawlor[30] with egg white lysozyme as a standard.

Electron microscopy. Granule pellets were overlayered with 30 to 40 μL human plasma and 30 to 40 μL 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After ten minutes at 25°C the granule-containing plasma plug was minced and submersed in fresh glutaraldehyde-cacodylate buffer for an additional 50 minutes. The minced plug was then washed and stored in 0.1 mol/L cacodylate buffer containing 7% sucrose.

Normal bone marrow was obtained in a heparinized syringe after informed consent by needle aspiration from the posterior iliac crest of a healthy volunteer (n = 1) and from patients undergoing routine staging of solid tumors (n = 2) or evaluation of lymphoid leukemia in remission and not receiving chemotherapy (n = 2). Bone marrow samples were similarly obtained from five untreated patients with myeloid leukemia (French-American-British classification M2) and a patient with a pancytopenic, preleukemic myeloproliferative disorder. Peripheral blood was collected from healthy volunteers in heparinized syringes by routine venipuncture after informed consent. Marrow and blood samples were fixed in 3% glutaraldehyde and 0.1 mol/L cacodylate, pH 7.35, and then rinsed in 0.1 mol/L cacodylate and 7% sucrose, pH 7.35, as described previously.[17]

Samples for myeloperoxidase staining were incubated 30 minutes in substrate medium consisting of 5 mgm DAB · 4 HCl (pfs grade, Sigma Chemical Co, St. Louis) in 10 mL 0.05 mol/L Tris-HCl buffer, pH 7.6, with 0.01% H_2O_2 (added immediately before use) as described previously.[31] Control samples were incubated in medium lacking H_2O_2. Some samples were also processed (for inhibition of MPO activity) in a complete substrate medium containing 10 mol/L KCN after a 30-minute preincubation in buffer containing 10 mol/L KCN.

Bone marrow and blood samples processed for staining of vicinal glycol–containing complex carbohydrates were incubated en bloc in α-amylase for removal of glycogen as described previously,[32] whereas this step was omitted for isolated cytoplasmic granules.

Samples processed for routine morphology and peroxidase cytochemistry were postfixed in 0.1 mol/L cacodylate-buffered 1% OsO_4, whereas those processed for vicinal glycol staining were not postfixed. All samples were routinely dehydrated and embedded in Spurr's low-viscosity medium. Thin sections of morphological and peroxidase-stained samples were collected on copper grids. Morphological specimens were examined after counterstaining with methanolic uranyl acetate (UA) and lead citrate (LC), whereas peroxidase-stained specimens were examined with and without counterstaining. Thin sections of specimens processed for vicinal glycol staining were collected on stainless steel grids and stained with a periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method as described previously.[32,33] Specimens were examined in a Philips 301 electron microscope or a Zeiss EM109 electron microscope at an accelerating voltage of 60 kV.

Granule secretion. Neutrophils (approximately 2 × 10^7/mL) were suspended at 37°C in PBS with 1 mol/L Ca^{++} and 1 mol/L Mg^{++} with or without 1 or 10 μmol/L calcium ionophore A23187 for 45 minutes. Control samples were incubated without ionophore or with ionophore in PBS lacking Ca^{++}. The cells were then centrifuged into a pellet, fixed, and examined ultrastructurally as described earlier. Granule fractions were isolated and assayed for MPO, β-glucuronidase, lysozyme, and vitamin B_{12} binding proteins as described earlier.

RESULTS

Isolation and properties of microgranules. Monodispersed granules of the PNS from normal human neutrophils were separated on an L-type Percoll density gradient which resulted in eight low-density isopycnic granule fractions as described previously by Rice et al.[9] The lowest density fraction, L1, was isolated and recentrifuged on an identical L-gradient, which resulted in remigration to its original characteristic isopycnic density (mean density, 1.046 ± 0.003 g/mL; n = 25 experiments). The majority of granules in the L1 fraction appeared homogeneously electron dense in UA-LC–stained morphological preparations (Fig 1). Morphometric analysis yielded a mean granule diameter of 0.15 ± 0.04, 0.14 ± 0.05, and 0.11 ± 0.05 μm for UA-LC–, DAB-, and PA-TCH-SP–stained specimens, respectively, and indicated that the L1 granule fraction consisted of a novel class of microgranules. In each granule preparation, 100 consecutively identified granules were scored in ran-

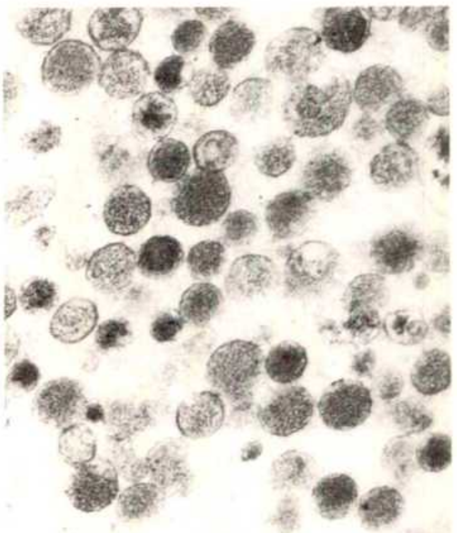

Fig 1. Ultrastructurally, the majority of granules in the L1 granule fraction appear diffusely stained with UA and LC and are variably electron dense. Bar, 1 μm.

domly selected photographs. All membrane-bound structures were scored, which would include tangential sections. Such scoring was used to prevent observer bias. Although repeat experiments did not clearly demonstrate a significant difference in diameter of PA-TCH-SP–stained preparations, the tendency for smaller size in these preparations may be related to the lack of OsO_4 postfixation before dehydration.

When the microgranules were reacted for peroxidase by using a DAB ultrastructural procedure, it was found that 34% to 50% (n = 4 experiments, 200 granules scored in each experiment) of the granules demonstrated intense staining (Fig 2A), thus indicating the existence of a DAB-reactive subclass of microgranules. The presence of DAB reactivity (30-minute incubation) at pH 7.6 rather than pH 9.7 alone indicated that the reactivity was not attributable to catalase alone, which was previously described as reacting at only the higher pH.[21] No DAB reactivity was observed in granules or neutrophils incubated 30 minutes without H_2O_2 or in the presence of 10 mmol/L KCN.

Biochemically, the microgranules of fraction L1 had a specific activity for peroxidase (units per milligram protein; electron donor, guaiacol) about five times less than that of typical, large primary granules (15.6 *v* 73.0, n = 6). This accounted for approximately 3% of the total granule-associated peroxidase activity of the neutrophil. The latency of MPO in this fraction was >95% after disruption of granules with 0.1% Triton X-100, which indicated that the peroxidase was localized within an intact, membrane-bound granule.

To identify the nature of the peroxidase activity associated with the microgranules, several characteristics of this fraction were investigated. First, the reduced minus oxidized

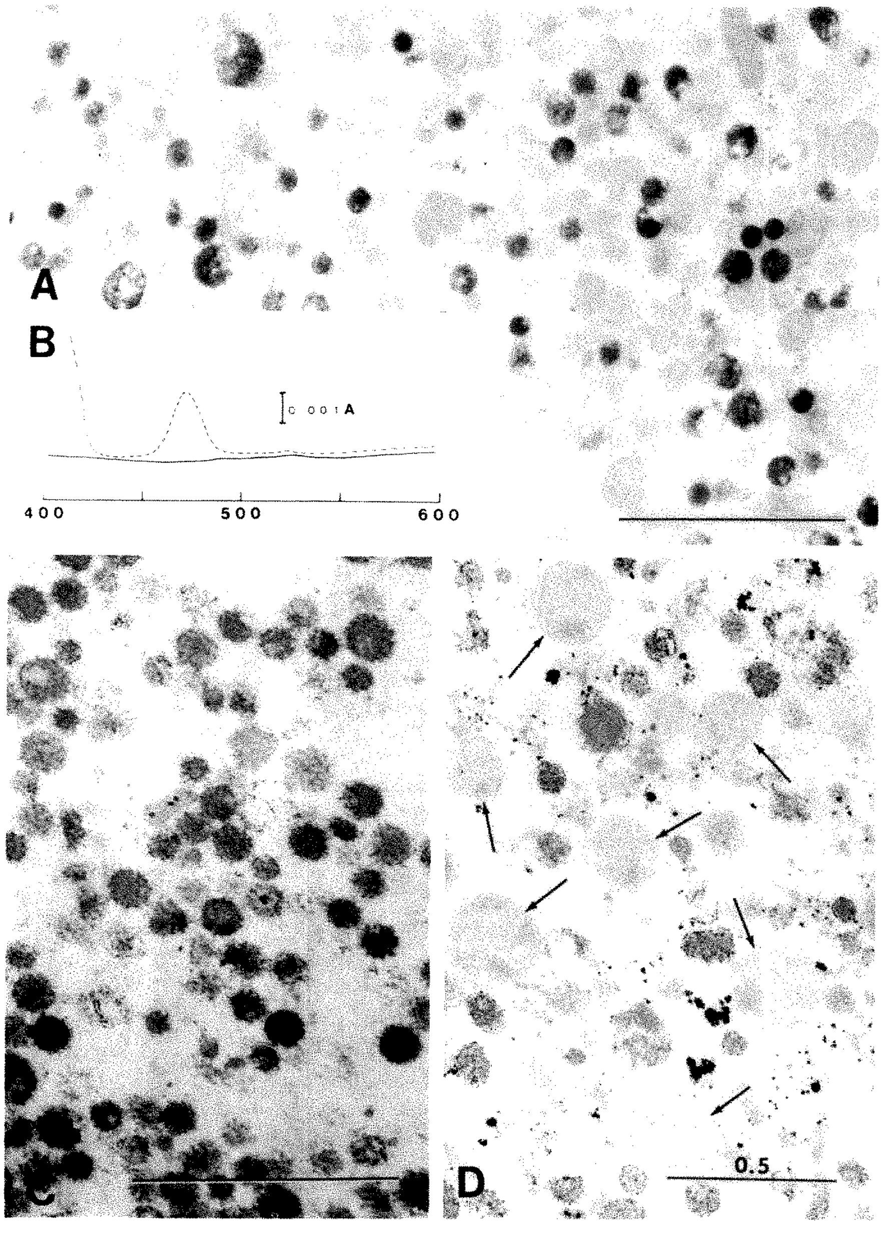

Fig 2. (A) Approximately 40% of microgranules in the L1 granule fraction demonstrate intense peroxidase reactivity after staining with DAB at pH 7.6 for 30 minutes. The thin section is not counterstained. Bar, 1 μm. (B). The reduced, minus-oxidized difference spectrum for the microgranule (L1) fraction displays a prominent absorption maximum at 472 nm, which indicates the presence of MPO. (C) PA-TCH-SP intensely stains vicinal glycol–containing complex carbohydrates in >97% of microgranules within the L1 fraction. The thin section is not counterstained. Bar, 1 μm. (D) After mixing granules from fraction L1 with granules from a typical primary granule fraction, H3, two populations of granules are seen. Microgranules demonstrate intense PA-TCH-SP staining, whereas typical primary granules are larger and essentially lack staining (arrows). The thin section is not counterstained. Bar, 0.5 μm.

difference spectrum for the microgranule fraction displayed a prominent absorption maximum at 472 nm, which indicated the presence of MPO (Fig 2B). Quantitation of the spectral data demonstrated that the microgranules contained 11 μg MPO/mg protein (range, 6 to 20; n = 3), which accounted for approximately 3% of the total granule-associated MPO on a per milligram protein basis (consistent with the enzymatic determination of MPO activity shown earlier). The absence of a trough at 430 nm due to absorption of oxidized MPO may be due to the presence of a b type cytochrome that has been reported to be colocalized with vitamin B_{12} binding protein[34]; however, because of technical difficulties in dealing with small absorbances in turbid suspensions, a definitive explanation is not possible. Interestingly, Vitamin B_{12} binding protein was concentrated in these lowest fractions,[35] and we have also confirmed the presence of the b type cytochrome in these same fractions by spectral analysis (data not shown). Second, a positive immunologic reaction for MPO was demonstrated on Ouchterlony plates when using a monospecific rabbit polyclonal anti-MPO (purified IgG fraction). Taken together, these results suggest that (*a*) the DAB reactivity seen in the ultrastructural preparations and that measured biochemically are due to the presence of MPO in the microgranule fraction and (*b*) this MPO appeared to be equivalent to that associated with typical primary granules.

Vicinal glycol–containing complex carbohydrates in the microgranule fraction stained strongly in greater than 97% of the granules when using the PA-TCH-SP procedure (Fig 2C). In marked contrast, typical, large primary granules in the denser granule fractions showed only very weak to negative PA-TCH-SP staining.[9,32] Furthermore, when isolated microgranules and larger, more dense, DAB-positive granules (fractions L1 and H3, respectively) were mixed and then stained by using the PA-TCH-SP procedure, each type of granule retained its characteristic staining pattern (Fig 2D). These results demonstrated that the peroxidase-positive microgranules were not fragments or tangential sections of the larger primary granules but belong to a newly recognized distinct subclass of MPO-containing granules that stain intensely for both peroxidase and periodate-reactive complex glycoconjugates.

Microgranules in intact normal cells. Intact neutrophil precursor cells of normal human bone marrow and mature peripheral blood neutrophils were examined ultrastructurally for the presence of microgranules. Small, intensely DAB- (Fig 3A, B) and PA-TCH-SP-reactive granules (Fig 3C) varying from 0.08 to 0.20 μm in diameter were observed

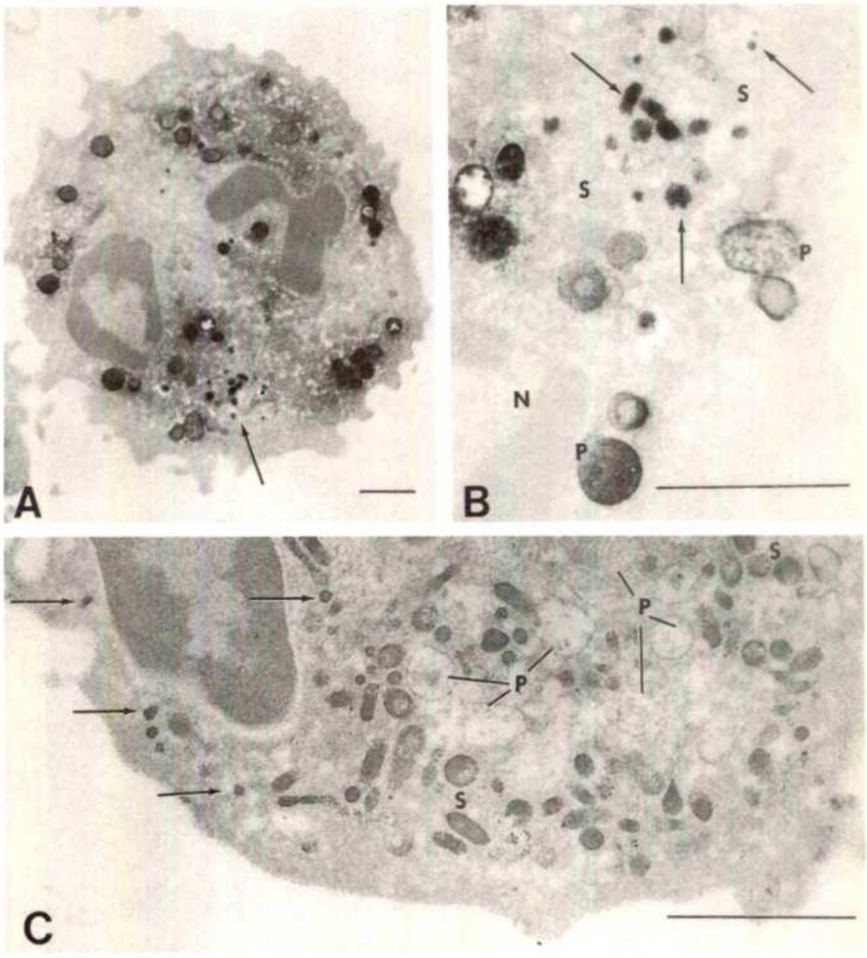

Fig 3. (A and B) Several DAB-reactive microgranules from a blood specimen are evident in a cluster (arrow, enlarged at right) in this segmented neutrophil. They are clearly smaller than larger DAB-reactive primary granules (P) and DAB-unreactive secondary granules (S); N, nuclei. The thin section is not counterstained. Bar, 1 μm. (C) PA-TCH-SP intensely stains microgranules (arrows) and secondary granules (S) of this neutrophil from a blood specimen but resulted in very weak to no staining of primary granules (P). The thin section is not counterstained. Bar, 1 μm.

at all stages of development beyond the myeloblast and appeared to correspond to the micrograules isolated from mature neutrophils. In the promyelocyte, these small granules were not readily apparent in that they were randomly dispersed and could not be easily distinguished from tangentially sectioned larger primary granules. In the myelocyte, the small granules were observed occasionally in clusters. This clustering phenomenon appeared to increase progressively with maturation. The small, DAB-reactive granules were more apparent in some profiles of fully mature neutrophils where they often appeared in clusters of three to more than ten granules. In the late (segmented) neutrophil, these clusters were observed in the central portion of the cell near the DAB-unreactive Golgi region as well as at the periphery of the cell.

Resistance of DAB-positive microgranules to secretion. Because the DAB-positive microgranules were found to be physically and chemically different from the large primary granules, the possibility they might exhibit differences in exocytosis in response to a secretory stimulus was examined. The calcium ionophore A23187 was chosen because this secretagogue has been reported to cause the differential secretion of secondary and primary granules in a dose-dependent manner (1 μmol/L and 10 μmol/L respectively).[36] Consistent with these previous reports, 1 μmol/L ionophore resulted in the loss of most DAB-negative granules and variable loss of DAB-positive granules, although DAB-positive microgranules consistently remained within the cell (Fig 4A). Again, consistent with previous reports,[37] exposure of the cells to 10 μmol/L ionophore resulted in virtually complete secretion of large (>0.20-μm-diameter) DAB-positive granules. In marked contrast the DAB-positive microgranules remained within the cells and frequently were redistributed radially to the subplasmalemmal region. These ultrastructural results were supported by Percoll density gradient centrifugation of granules prepared from 10 μmol/L ionophore-treated neutrophils in which the only visibly apparent granule fractions were the two lowest density granule fractions, L1 and L2.

Additional support for the selective retention of these MPO-containing microgranules was obtained by quantitating the distribution of MPO in isolated granule fractions from untreated and treated cells. In untreated cells, 70% of the total MPO activity was associated with the larger, high-density granules found in fractions H1 to H5, 22% with the intermediate-density fractions (L3 to L8) and 8% with the two lowest density fractions (L1 and L2). After treatment with 10 μmol/L ionophore, only fractions L1 and L2 retained significant amounts of their original MPO activity (Fig 4B); fraction L1 retained 96% ± 36% (n = 5), whereas fraction L2 retained 36% ± 10% (n = 5). In other experiments (n = 2) using neutrophils from different donors, treatment with 10 μmol/L ionophore resulted in the retention of 104% and 63% of the β-glucuronidase activity in the

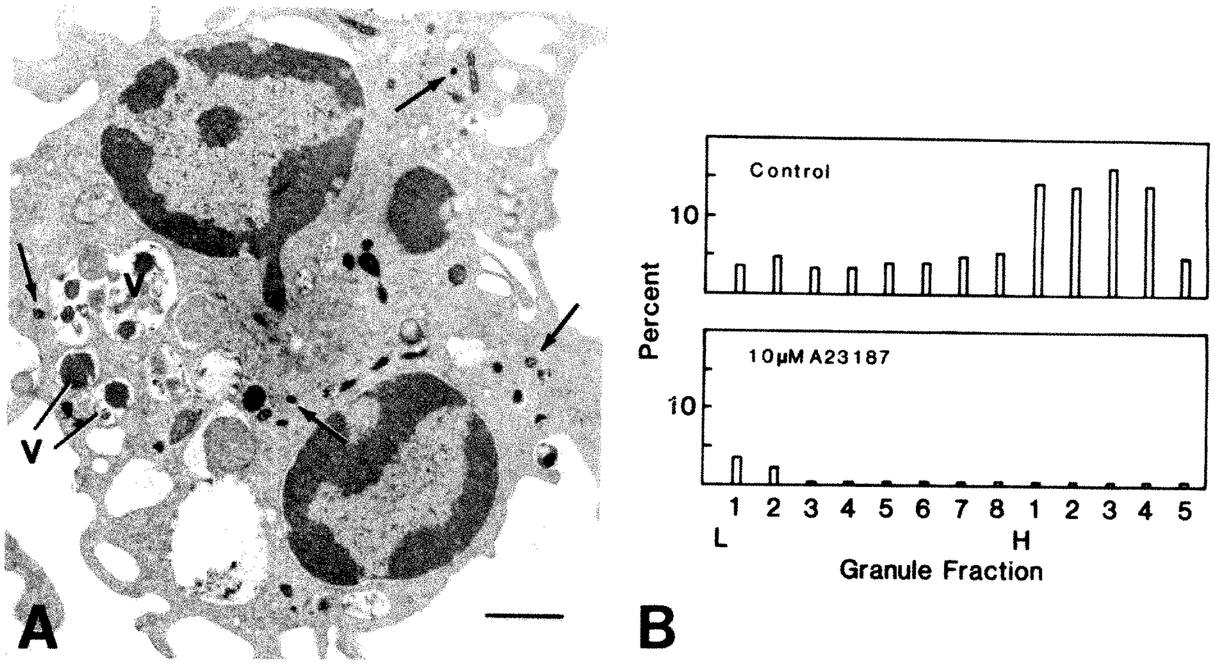

Fig 4. (A) Approximately 20% and 80% of neutrophil profiles contain predominantly DAB-reactive microgranules (arrows) after exposure to 1 and 10 μmol/L of calcium ionophore A23187 respectively (1 mmol/L sample illustrated). DAB-negative granules and large DAB-positive granules have degranulated. The granule matrices of some larger primary granules are evident in vacuoles (V) or channels into which they have degranulated. Less dense secondary granules are not evident and have presumably degranulated. The thin section is lightly counterstained with UA and LC. Bar, 1 μm. (B) Granules from untreated neutrophils (control) or from neutrophils after treatment with 10 μmol/L calcium ionophore A23187 for 45 minutes at 37°C (10 μmol/L A23187) were separated by using L and H Percoll gradients to prepare fractions L1 to L8 and H1 to H5, respectively, as previously described.[9] Granule fractions were isolated and assayed for their content of MPO. The amount of MPO activity in each fraction is expressed as a percentage of the sum of MPO activity in all fractions of control samples (ordinate). The abscissa identifies the individual granule fractions.

L1 fraction compared with control samples. Similarly, 70% and 72% of lysozyme (n = 2 experiments) was retained in the L1 fraction after 10 μmol/L A23187 compared with control values. In contrast, only 6% and 0% of vitamin B_{12} binding protein activity (n = 2 experiments) was retained in the L1 fraction after 10 μmol/L A23187 compared with simultaneous controls. The percent retention of a particular marker was based on the following control values (mean ± SD) for the L1 granule fraction derived from 2×10^8 neutrophils in both the control and ionophore-treated experiments: MPO (2.6 ± 0.6 units, n = 5), β-glucuronidase (2.0 ± 0.7 mU, n = 4), vitamin B_{12} binding proteins (28,260 ± 4,820 cpm bound, n = 3), and lysozyme (84 ng and 76 ng, n = 2). The results obtained with MPO indicated that although DAB-positive microgranules were localized primarily in fraction L1, a portion was also associated with fraction L2 (Fig 4B). Ultrastructurally, the DAB+ microgranules found in fraction L2 had identical morphometric, DAB, and PA-TCH-SP staining properties as those found in fraction L1. Granules from fraction L1 were isolated from 10 μmol/L ionophore-treated cells and were processed for ultrastructural staining using DAB. Of the remaining granules, 71% ± 8% (100 granules scored, n = 3) were DAB-positive. Mitochondria were more prominent in these preparations and were excluded from granule scoring. Thus, the DAB-positive microgranules were highly resistant to secretion relative to the larger, more typical primary granules (fractions H1 to H4), thereby demonstrating differences in secretory control between the two granule types.

Microgranules in intact leukemic myeloblasts and myelodysplastic neutrophils. Ultrastructural studies were performed on intact leukemic myeloblasts from the bone marrow of five myeloid leukemia patients (French-American-British classification M2). In three cases, the predominant granule type observed was a small, DAB- and PA-TCH-SP–reactive granule varying from 0.08 to 0.20 μm in diameter (Fig 5A, B). Auer rods, which were present in two of the patient samples, were uniformly and intensely stained with DAB and PA-TCH-SP (Fig 6). Examination of leukemic cells from two other patients with acute myelogenous leukemia (M2 classification) revealed a more heterogeneous population of DAB-positive granules; however, distinct microgranule profiles were evident.

Myelodysplastic neutrophils from a patient with a pancytopenic preleukemic syndrome (1 year prior to developing myeloid leukemia) appeared grossly abnormal in morphological preparations and completely lacked DAB-unreactive granules. Two distinct granule populations were evident in these cells, a homogeneous electron-dense and DAB-reactive large primary granule (0.25 to 0.40 μm in diameter) and a small (0.08 to 0.20 μm in diameter) electron-dense, DAB- and PA-TCH-SP–positive granule (Fig 7A, B). The small granule type outnumbered the larger primary granules and often appeared in clusters as seen in normal neutrophils. The increase in microgranules was also observed in promyelocytes and myelocytes from this patient.

DISCUSSION

The present studies identify a peroxidase-containing microgranule population in human neutrophils that is distinct from previously described primary granules. Small granule profiles have been previously identified in intact cells at the early promyelocyte stage of development.[1] Similarly, DAB-positive microgranule clusters have been observed in

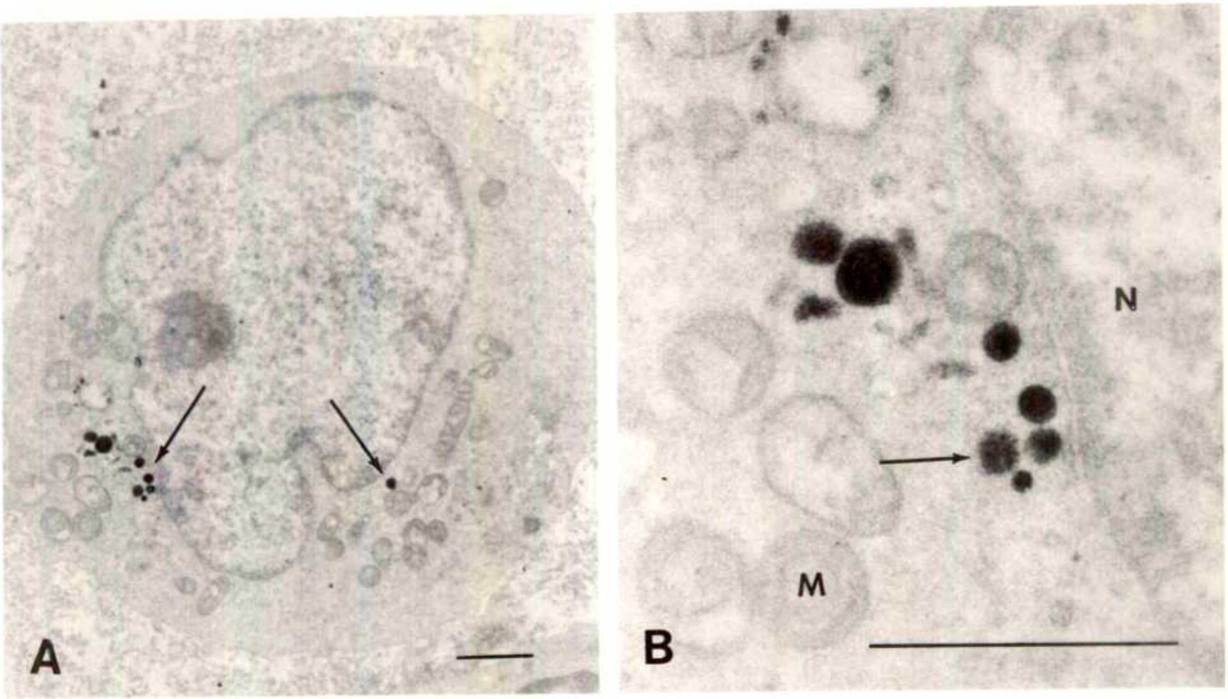

Fig 5 A, B. This leukemic myeloblast from a marrow specimen contains a cluster of small granules (arrows, enlarged in B) that stain intensely with DAB. N, nucleus; M, mitochondria. The thin section is not counterstained. Bar, 1 μm.

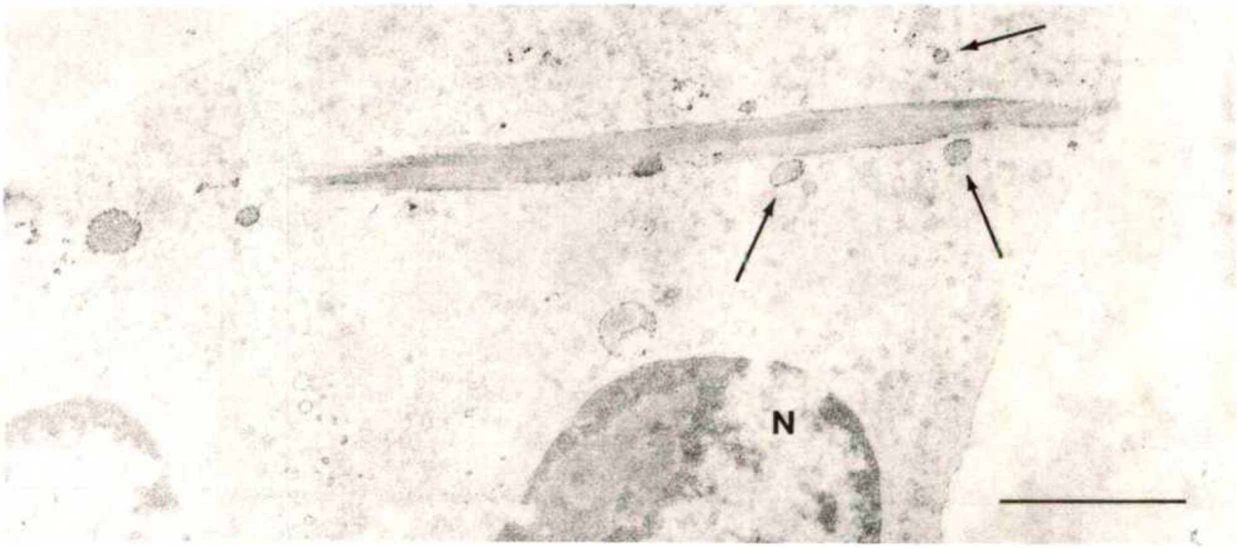

Fig 6. PA-TCH-SP intensely stains micrugranules (arrows) in this leukemic myeloblast from a marrow specimen. Many of the micrugranules are adjacent to an intensely stained Auer rod; N, nucleus. The thin section is not counterstained. Bar, 1 μm.

segmented neutrophils[2,22]; however, previous studies have not isolated the granule, defined the enzyme responsible for the DAB positivity, or examined cytochemical, physical, or secretory features of the granule. The present study establishes the existence of MPO in isolated micrugranules by using cytochemical, biochemical, and immunologic methods and provides a method for concentrating these granules from cells after ionophore-induced secretion. Biochemical analysis of the concentrated micrugranule fraction indicates that they resemble primary granules in their content of β-glucuronidase and lysozyme and lack of vitamin B_{12} binding protein activity. The DAB reactivity of micrugranules and larger

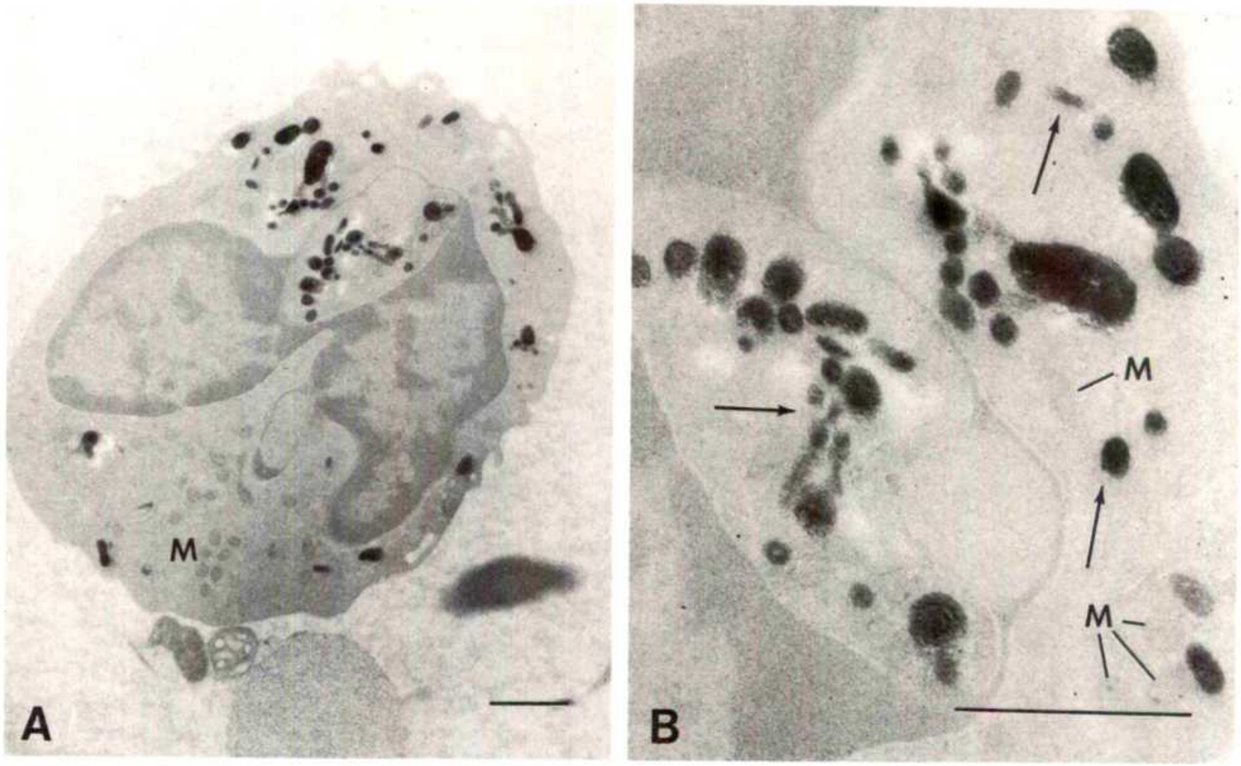

Fig 7 A, B. This segmented neutrophil from a marrow specimen of a patient with a myeloproliferative disorder contains predominantly DAB-reactive micrugranules (arrows, enlarged in B) and a few larger DAB-reactive primary granules. DAB-unreactive secondary granules are markedly decreased or absent. Numerous mitochondria (M) that lack DAB reactivity (30-minute incubation, pH 7.6) are evident. The thin section is not counterstained. Bars, 1 μm.

primary granules demonstrate that DAB reactivity and MPO content span the entire gamut of neutrophil granule size and density.

Previously described primary granules[1-5] are two to three times the size of microgranules and have considerably higher buoyant densities than do the microgranules. The intense PA-TCH-SP staining of vicinal glycols in microgranules is clearly distinct from the weak to absent staining of larger primary granules and demonstrates that microgranules also differ in content from previously described primary granules.[32] Moreover, the DAB-positive microgranules are resistant to 10 μmol/L A23187 ionophore–induced secretion, which demonstrates that these granules are under secretory control distinct from typical primary granules that were secreted under these conditions. Hence, the differences in size, density, carbohydrate content, and resistance to secretion of the DAB-positive microgranules identify them as a unique population of granules for which the terms primary or azurophil may be inappropriate, although the latter terminology has been used for all neutrophil granules with peroxidase activity.

Neutrophil granules contain several potentially DAB-reactive substances. In addition to containing at least three forms of MPO,[14,15] neutrophils also contain catalase-reactive peroxisomes,[21] which may correspond in size to the microgranules in this study. Although some of the microgranules in the lowest density fraction may, in fact, be peroxisomes, the DAB reactivity of the granules at pH 7.6, the presence of biochemically, spectrally, and immunologically demonstrable MPO in these granules, and the great abundance of these granules in some leukemic myeloblasts would appear to exclude this possibility for the DAB-reactive microgranule described in this study. Alternatively, a b-type cytochrome has been identified as a component of secondary granules and/or other small, low-density granules,[34] and cytochromes are known to be DAB-reactive.[38] The b-type cytochrome has been shown to translocate to the plasma membrane after stimulation of the cell with either a phorbol diester or the calcium ionophore A23187[34]; however, we found that stimulation of neutrophils by the ionophore did not result in secretion of the DAB-reactive microgranules. Furthermore, the abundance of similar granules in leukemic cells (presumably lacking secondary and/or cytochrome b–containing granules) also suggests that a b-type cytochrome is not responsible for the staining of microgranules in this study. Thus, the cytochemically demonstrable DAB reactivity is most consistent with the localization of MPO identified biochemically, spectrally, and immunologically in this granule fraction.

The presence of DAB-reactive microgranules in leukemic myeloblasts indicates the synthesis of at least some of these granules early in myeloid development. Conversely, the lack of DAB reactivity in Golgi lamellae and the endoplasmic reticulum beyond the myelocyte stage of development indicates that the granules are not synthesized in late neutrophils. Recent studies have suggested that microgranules are synthesized in late promyelocytes after synthesis of larger primary granules[22]; however, this hypothesis does not readily account for the previously observed synthesis of small granules (0.10 to 0.25 μm in diameter) in the earliest promyelocytes[1] and the observation in this study of abundant microgranules in some leukemic myeloblasts. Similarly, cultured myeloid leukemic HL-60 cells contain predominantly small MPO-positive granules of comparable density to the microgranules reported in this study.[9] Alternatively, the microgranules could be synthesized throughout the promyelocyte stage of development or exist as subpopulations being synthesized in early and late promyelocytes, respectively.

The microgranules were redistributed into clusters, observed both in normal and myelodysplastic neutrophils in this study. Similar clusters of DAB-reactive small granules have been observed in monocytes and have been taken to indicate functional subcompartmentalization.[39] Although clustering may represent worm-like structures in monocytes, the lack of such elongated profiles in the present study of intact neutrophils and isolated granules or in previous high-resolution electron microscopic studies of microgranules[22] indicates differences in the small DAB-reactive granules of monocytes and neutrophils. The clustering phenomenon presumably has functional significance in neutrophils and may occur in preparation for endocytosis or as a result of this process. The extreme resistance of microgranules to secretion is similar to that observed with residual bodies or secondary lysosomes[40] and may indicate prior involvement in an endocytic event. However, the lack of inclusions or ultrastructural heterogeneity within the microgranule matrix would argue against this possibility. Alternatively, the DAB-positive microgranules could represent fragmented primary granules; however, the intense PA-TCH-SP reactivity of the microgranules and the virtual lack of similar reactivity in primary granules does not support this hypothesis. The sequence of granule production has in the past correlated with resistance to granule secretion in that tertiary granules are secreted most readily,[18] followed by secondary granules[34] and primary granules. The observation of small granule profiles in the earliest promyelocytes[1] and leukemic myeloblasts in this study would suggest that these are among the first granules synthesized and the last secreted if at all.

NOTE ADDED IN PROOF

Recently, White and Krumwiede[41] have ultrastructurally demonstrated failure of peroxidase positive "vesicles" and granules to fuse with giant lysosomes in neutrophils from patients with Chédiak-Higashi Syndrome. Many of these granules were of similar size to microgranules reported in this study. We would suggest that their observation represents in vivo resistance to secretion comparable to that observed in vitro in our study.

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The Metabolite 1α,25-Dihydroxyvitamin D_3 Enhances Lymphokine-Mediated Activation of the Oxidative Metabolism of the U937 Cell Line and Phosphorylation of a 48-kD Respiratory Burst–Associated Protein

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U937 cells respond to a variety of stimuli with increased differentiation as manifested by reduced growth, increased adherence, increased expression of several surface receptors, and increased capacity for phagocytosis and formation of reactive oxygen intermediates. In the present study the effects of lymphocyte conditioned media, recombinant interferon-γ (IFN-γ), and 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on the ability to form reactive oxygen intermediates by U937 cells were measured by using the luminol-dependent luminescence (LDL) assay. Neither $1,25(OH)_2D_3$ alone nor IFN-γ alone enhanced competence for phorbol myristate acetate–stimulated LDL. Cells were capable of moderate LDL after exposure to lymphocyte conditioned media, and this was enhanced by $1,25(OH)_2D_3$ (10^{-8} mol/L) and other vitamin D metabolites at higher concentrations. This effect was not secondary to accelerated production of myeloperoxidase, which is important in the LDL assay. Enhanced phorbol myristate acetate-stimulated phosphorylation of a 48-kd substrate was observed in ^{32}P-labeled intact cells treated with $1,25(OH)_2D_3$ alone or in combination with IFN-γ. Treatment of cells with IFN-γ or lymphocyte conditioned media did not alter phosphorylation. These results support the concept that $1,25(OH)_2D_3$ plays a role in phagocyte differentiation and activation beyond the effects of lymphokines. Protein kinase C–mediated phosphorylation reactions may be necessary for the ability of U937 cells to reduce O_2 and required for maximal activity under some conditions of incubation.

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THE U937 CELL LINE is a monoblastic phagocyte precursor derived from a histiocytic lymphoma cell line.[1] U937 cells have become an important model for the study of phagocyte differentiation because they respond to a wide and surprising variety of biologic modifiers.[2]

The active metabolite of vitamin D 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has a profound effect on calcium homeostasis.[3] We and others have previously demonstrated that $1,25(OH)_2D_3$ enhances the differentiation of U937 cells as manifested by increased phagocytosis and expression of CR1 receptors.[4,5] The metabolite $1,25(OH)_2D_3$ has an additive effect with one or more lymphokines such that this combination induces adherence of the U937 cell line to plastic as well as expression of CR3 receptors.[6] Both $1,25(OH)_2D_3$ and lymphokine enhance the ability of U937 cells to reduce oxygen.[4,5,7,8] The present studies were undertaken to define the interaction of $1,25(OH)_2D_3$ and lymphokines on the differentiation of U937 cells, with emphasis on formation of reactive oxygen intermediates.

METHODS

Reagents

The metabolites $1,25(OH)_2D_3$ and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) were a gift from Milan Uskokovic, PhD (Hoffman-LaRoche, Nutley, NJ). These metabolites were dissolved in 95% ethanol and diluted to give a final concentration of <0.1% (vol/vol) ethanol. In all experiments appropriate controls were performed to exclude an ethanol effect. The metabolite 25-hydroxyvitamin D_3 ($25(OH)D_3$) was a gift from the Upjohn Pharmaceutical Co (Kalamazoo, MI). Superoxide dismutase (SOD, Sigma Chemical Co, St Louis) was used at a final concentration of 150 U/mL. Sodium azide (Fisher Scientific Co, Fair Lawn, NJ) was used at a final concentration of 1 mmol/L. Recombinant interferon-γ (IFN-γ; Meloy Laboratories, Springfield, VA) was used to induce cell differentiation at a concentration of 1,000 U/mL. Phorbol myristate acetate (PMA, Midland Chemical Co, Brewster, NY) was diluted in Me_2SO and used at a final concentration of 100 ng/mL.

Cell Preparation and Assay

Culture conditions. U937 cells were maintained in continuous culture by passage every three days in medium consisting of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium (1:1; GIBCO, Grand Island, NY). The media were supplemented with 5% fetal bovine serum (GIBCO), 15 mmol/L HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). These were considered control cells. The metabolite $1,25(OH)_2D_3$ reduced U937 cell growth[4] but did not affect viability. When experimental variables were added, the cells were resuspended at a final concentration of 10^6 cells/mL. The cells were incubated at 37°C in humidified 5% CO_2.

Lymphocyte conditioned media. These media were prepared from lymphocytes obtained from healthy human volunteer donors. Whole heparinized blood was placed on a Plasmagel (Cellular Products Inc, Buffalo) sedimentation gradient. The white cell layer was then placed over Ficoll-Hypaque as previously described.[9] The mononuclear band was washed twice at 100 g in phosphate-buffered saline. The cells were then placed in a 100 × 20-mm Petri dish (Corning Glass Works, Corning, NY) medium consisting of α-modified minimal essential medium (α-MEM, GIBCO) and the monocytes allowed to adhere for two hours. The nonadherent cells were then removed and counted by the Zaptoglobin lysis technique (Coulter Diagnostics, Hileah, FL). The cells were then suspended in α-MEM at a final concentration of 1×10^6 cells/mL in 5% pooled human serum. Phytohemagglutinin (PHA, Sigma) was added at a concentration of 5 μg/mL. The cells were incubated at 37°C in humidified 5% CO_2 for three days. The cells were then centrifuged at 250 g for ten minutes. The supernatant was collected and filtered.

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Aliquots were frozen at 0°C until use. Lymphocyte conditioned media were used at concentrations of 10% to 20% (vol/vol); the lymphokine preparation was only reduced in concentration in preparations that affected cell viability. Controls included lymphocytes isolated under identical conditions with PHA added to the lymphocyte conditioned media after the three-day incubation.

Luminol-Dependent Luminescence

Measurements of chemiluminescence were made with an ATP spectrophotometer (Model 3000, SAI Technology Co, San Diego). Light intensity was monitored continuously by using a strip chart recorder (Fisher Recordall, Pittsburgh) as previously described.[10] Luminescent counts were also obtained at one-minute intervals from the digital readout of the spectrophotometer. The sensitivity was the same in all experiments (9.4). The temperature of the counting chamber was 31°C. The cell suspension was pelleted, media discarded, and cells resuspended in Hanks' balanced saline solution (HBSS) at a final concentration of 5×10^6 cells/mL. These cells remained in a 37°C water bath until use. Background counts were obtained by adding 1.0 mL of a 10^{-4} mol/L solution of luminol (Sigma) prepared in 1% bovine serum albumin to 1.0 mL of HBSS. Resting cell luminescence was obtained by adding 1.0 mL luminol to 1.0 mL of the cell suspension. Counts were obtained over a 15-minute interval. PMA (100 ng/mL) was used as a stimulus. Counts were recorded at one-minute intervals until peak luminescence was reached. Samples were examined in duplicate.

Myeloperoxidase Assay

The myeloperoxidase activity of U937 cells was determined by the *O*-dianisidine reaction as previously described.[11]

Endogenous Substrate Phosphorylation in Intact Cells

Substrate phosphorylation was determined as previously described.[12-15] After six days of exposure to the various agents, the cells were centrifuged and resuspended in Eagle's minimal essential medium with Earle's salt but without glutamine or phosphates (GIBCO) for 30 minutes. A total of 3×10^6 cells were incubated with ^{32}P, 0.25 mCi, for two hours, after which the cells were centrifuged and the supernatant was removed. After resuspension in fresh medium, the cells were exposed to vehicle (Me_2SO, 0.01%) or PMA (100 ng/mL) for ten minutes. The reaction was terminated by adding a one-third volume of a solution containing 9% (wt/vol) sodium dodecyl sulfate, 15% (wt/vol) glycerol, 30 mmol/L Tris-HCl, pH 7.8, 0.05% (wt/vol) bromophenol blue, and 6% vol/vol) mercaptoethanol. The mixture was heated to 110°C for three minutes. The samples were subjected to one-day polyacrylamide gel electrophoresis (PAGE) as described by Laemmeli.[15] The stacking and running gels were 3% and 10% acrylamide, respectively. After staining and destaining, the gels were treated with 1 mol/L potassium hydroxide for one hour at 56°C. This procedure allowed optimal visualization of PMA-dependent substrate phosphorylations. After treatment for one hour in the destaining solution, the gels were dried, and autoradiography was performed. Concurrent electrophoresis of molecular weight (mol wt) standards allowed assignments of approximate mol wts to the phosphorylated substrates. Incorporation of ^{32}P into endogenous substrates was quantitated by densitometric analysis of the autoradiogram. The autoradiograms were scanned at 600 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

RESULTS

Effects of $1,25(OH)_2D_3$ and Lymphocyte Conditioned Media on the O_2 Metabolism of U937 Cells

U937 cells were exposed to a variety of agents believed to be of importance in their differentiation. These included lymphokine conditioned media (harvested from cells stimulated with PHA), monoclonal IFN-γ, and $1,25(OH)_2D_3$ and other vitamin D metabolites.

Table 1 demonstrates the effects on cells examined five days after incubation with lymphocyte conditioned media alone, $1,25(OH)_2D_3$ alone, and $1,25(OH)_2D_3$ plus lymphocyte conditioned media. Cells exposed to lymphocyte conditioned media alone demonstrated an increase in luminol-dependent luminescence (LDL) in response to PMA. Cells incubated with lymphocyte conditioned media plus $1,25(OH)_2D_3$ showed a dramatic increase in LDL, whereas $1,25(OH)_2D_3$ alone did not promote LDL.

Table 1. Peak LDL (cpm × 10^3)

Conditions	Mean	SD	Increase Over Control (fold)	Number of Experiments
Background	2	.50		14
Control	2	.50		14
CM	8.5	.50	4.2	14
CM + $1,25(OH_2D_3$ (10^{-8} mol/L)	82	15.0	41	14
INF-γ, 1,000 U/mL	2	.50	0	14
INF-γ + $1,25(OH)_2D_3$	45	12.0	22.5	14
CM + $24,25(OH)_2D_3$ (10^{-6} mol/L)	25	.50	12.5	3
CM + $25(OH)_2D_3$ (10^{-6} mol/L)	27	.50	13.5	3
$1,25(OH)_2D_3$	2	.50	0	14
$24,25(OH)_2D_3$	2	.50	0	3
$25(OH)D_3$	2	.50	0	3
CM + $1,25(OH)_2D_3$ + SOD	2	.20	0	10
CM + $1,25(OH)_2D_3$ + azide	18	.45	9	3

The effects of incubation of U937 cells on competence for LDL stimulated by PMA (100 ng/mL). The cells were in culture for five days before the experiments were conducted. Background refers to emission of light by media. The control for these experiments was conditioned media harvested from lymphocytes not exposed to PHA. SOD was used at a concentration of 300 U/mL. One-millimolar azide was used.

Abbreviation: CM, lymphocyte conditioned media, 20% vol/vol.

Effects of IFN-γ and $1,25(OH)_2D_3$ on O_2 Metabolism of U937 Cells

IFN-γ is a lymphokine believed critical to the differentiation and activation of monocytic phagocytes.[16] The effects of IFN-γ were compared with lymphocyte condition media, as shown in Table 1. LDL was not observed among cells treated with INF-γ alone. Treatment of cells with INF-γ plus $1,25(OH)_2D_3$ for five days enhanced competence for LDL but of less magnitude than cells treated with lymphocyte conditioned media and $1,25(OH)_2D_3$ (Table 1).

To determine whether IFN-γ resulted in the same degree of U937 cell differentiation after varied periods of incubation, the same comparisons were made among cells incubated for zero to seven days (Fig 1). At each time point the effects observed with lymphocyte conditioned media plus $1,25(OH)_2D_3$ were equivalent to or greater than the combination of $1,25(OH)_2D_3$ and IFN-γ.

Effect of Other Vitamin D Metabolites on the O_2 Metabolism of U937 Cells

The vitamin D metabolite most important for calcium homeostasis is $1,25(OH)_2D_3$.[3] Receptors for $1,25(OH)_2D_3$ have been demonstrated in human monocytes.[17] The metabolites $24,25(OH)_2D_3$ and $25(OH)D_3$ generally have lower affinity for such receptors than the active metabolite $1,25(OH)_2D_3$.[3] The effects of $1,25(OH)_2D_3$ were compared with other metabolites of vitamin D (Table 1). Neither $24,25(OH)D_3$ nor $25(OH)_2D_3$ promoted maximal competence for LDL, even when concentrations as high as 10^{-6} mol/L were used.

Effect of Adherence of U937 Cells on LDL

Lymphokine plus $1,25(OH)_2D_3$ increases U937 cell adherence from 3% to 57%.[6] To determine the role of adherence in LDL, adherent cells were removed with a rubber policeman, and PMA-stimulated LDL was measured relative to an equivalent number of cells in suspension. As shown in Table 2, LDL was greater among adherent cells than cells in suspension, which suggests that competence for LDL is a parameter of differentiation correlated with adherence.

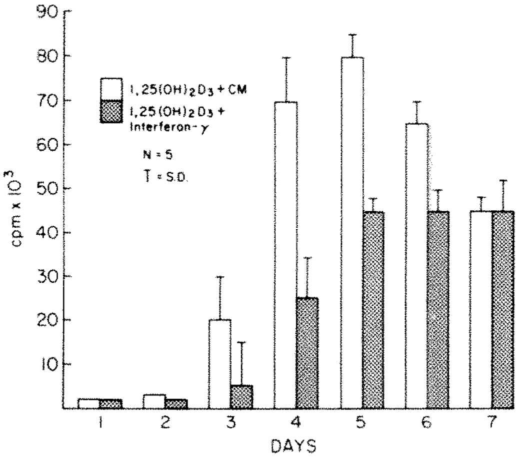

Fig 1. Comparison of $1,25(OH)_2D_3$ plus lymphocyte conditioned media or IFN-γ over seven days. Bars represent the mean and brackets the SEM of three to four separate experiments. The cells were stimulated with 100 ng/mL PMA.

Mechanism(s) of Oxygen Reduction by U937 Cells

LDL results from the interaction of luminol with several reactive oxygen intermediates.[18-21] As shown in Table 1, all light emission was eliminated by the addition of SOD to PMA-stimulated cells, thus indicating that superoxide was a critical species for this reaction. Light emission was inhibited to a lesser extent by sodium azide, which implied that a proportion of light could be attributed to the formation of hypochlorous acid, as has been reported for human neutrophils.[20] All U937 cells examined after differentiation expressed myeloperoxidase in equivalent concentrations (data not shown). These results suggest that differences in LDL related directly to the ability to form superoxide anion.

Phosphorylation of Endogenous U937 Substrates

Several recent studies have focused on protein kinase C activation and substrate phosphorylation during PMA-stimulated formation of reactive oxygen intermediates.[22-26] Specifically, PMA-stimulated phosphorylation of a substrate with an approximate mol wt of 43 to 48 kD has been associated with superoxide formation,[22,25,26] which suggests that these events are important in signal transduction. We examined the effects of $1,25(OH)_2D_3$ on phosphorylation reactions in intact cells stimulated with PMA. U937 cells incubated with 10^{-8} mol/L $1,25(OH)_2D_3$ for six days were labeled with ^{32}P and treated with PMA under conditions identical to those used to measure competence for LDL (Fig 2); longer exposure to PMA did not change these results (data not shown). When compared with control cells, $1,25(OH)_2D_3$ exposure increased PMA-dependent phosphorylation of 68- and 48-kD substrates (Fig 2). PMA-dependent phosphorylation of a 17-kD substrate was not altered by $1,25(OH)_2D_3$ pretreatment. Exposure to $1,25(OH)_2D_3$ also enhanced the non–PMA-dependent phosphorylation of a 74-kD substrate. We also examined phosphorylation in cells treated with IFN-γ, lymphocyte conditioned media, or $1,25(OH)_2D_3$ in the presence and absence of

Table 2. Peak LDL (cpm × 10^3)

Experiment	Adherent Cells	Nonadherent Cells	Percentage of Control
1	104	54	52
2	124	60	48
3	84	64	76
4	75	50	66
Mean	93	57	60.5

Cells were cultured for five days in the presence of (20%) lymphocyte conditioned media and $1,25(OH)_2D_3$ (10^{-8} mol/L), after which time nonadherent cells were prepared by gentle removal with a rubber policemen, adjusted for viability, and compared with adherent cells stimulated with PMA (100 ng/mL). Results from four separate experiments are given, and LDL of nonadherent cells is presented as a percentage of adherent cells.

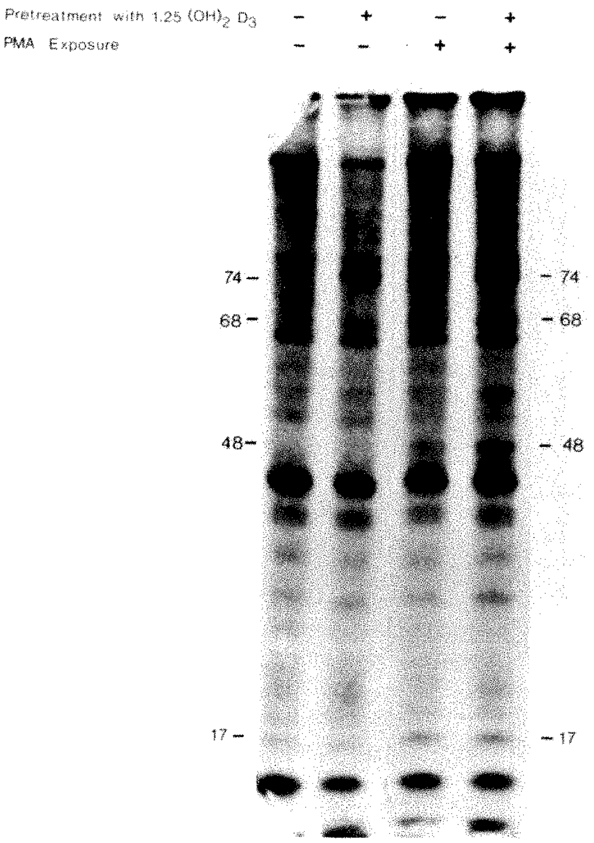

Fig 2. The effect of 1,25(OH)$_2$D$_3$ treatment on PMA-stimulated phosphorylation in ^{32}P-labeled U937 cells. After a six-day incubation with 10^{-8} mol/L 1,25(OH)$_2$D$_3$ or vehicle, the cells were labeled with ^{32}P for two hours. The ^{32}P-labeled cells were exposed to 100 ng/mL PMA for ten minutes. PMA-stimulated phosphorylation was analyzed as described in Methods. Identical results were obtained when a saturating dose of PMA was used. These data are representative of three separate experiments.

Table 3. Effects of Differentiation on 48-kD Substrate Phosphorylation of Intact Cells

Pretreatment Conditions	PMA Exposure	Substrate (48 kD) Phosphorylation (Arbitiary Units)
Vehicle (control)	−	0.53 ± 0.01 (100)*
Vehicle	+	0.68 ± 0.03 (128)
1,25(OH)$_2$D$_3$, 10^{-8} mol/L	+	0.81 ± 0.01 (154)
INF-γ, 1,000 U/mL	+	0.68 ± 0.04 (128)
1,25(OH)$_2$D$_3$ + IFN-γ	+	0.88 ± 0.14 (166)
CM, 10%	+	0.59 ± 0.13 (111)
1,25(OH)$_2$D$_3$ + CM†	+	0.89 ± 0.09 (168)

A total of 0.3×10^6 cells/mL were treated for six days under the conditions listed above. After the treatment period the cells were labeled with ^{32}P and exposed to vehicle or PMA (100 ng/mL) for ten minutes. The samples were subjected to PAGE followed by autoradiography as described in Methods. The 48-kD substrate phosphorylation was quantitated by densitometric analysis. These data represent the mean and SEM of three separate experiments.

*Percentages of 48-kD substrate phosphorylation in vehicle-treated cells not exposed to PMA are in parentheses.

†These results reflect the mean 48-kD phosphorylation of adherent and suspended cells examined on four separate occasions.

IFN-γ (Table 3, Fig 3). PMA-stimulated, 48-kD substrate phosphorylation was detected under all conditions of incubation; cells exposed to lymphocyte conditioned media or IFN-γ were not significantly different from cells treated with vehicle. In cells treated with 1,25(OH)$_2$D$_3$ alone or in combination with IFN-γ, PMA-stimulated, 48-kD substrate phosphorylation was increased relative to the other conditions examined. Concomitant exposure of cells to 1,25(OH)$_2$D$_3$ and lymphocyte conditioned media did not enhance 48-kD phosphorylation beyond that observed with 1,25(OH)$_2$D$_3$ alone (Table 3). Furthermore, no difference in 48-kD phosphorylation was detected when adherent and suspended cells (exposed to the combination of 1,25(OH)$_2$D$_3$ and lymphocyte conditioned media) were compared as described in Table 2 (data not shown). The background (PMA-independent substrate phosphorylation) was similar in all conditions examined.

DISCUSSION

The U937 cell line has provided a unique model for study of the differentiation and activation of phagocytic cells. By using this cell line it has been possible to demonstrate cell differentiation in response to a wide variety of substances.[2] The active metabolite of vitamin D is 1,25(OH)$_2$D$_3$.[3] Receptors for 1,25(OH)$_2$D$_3$ have been demonstrated in monocytic phagocytes.[17] We have previously shown that 1,25(OH)$_2$D$_3$ enhances differentiation of U937 cells as demonstrated by expression of complement receptors.[4] These effects are most dramatic when 1,25(OH)$_2$D$_3$ is combined with lymphokines.[6]

Differentiation of U937 cells also has been associated with the development of the ability of these cells to reduce oxygen, a function important if not critical for microbicidal and tumorcidal activity of mononuclear phagocytes.[27] U937 cell differentiation is associated with competence for nitroblue tetrazolium dye reduction,[5] formation of hydrogen peroxide,[7,8] and expression of LDL.[28]

The present studies were undertaken to extend our earlier observations[4,6] regarding the interaction of lymphokines and 1,25(OH)$_2$D$_3$. Consistent with these results, we observed maximal competence for LDL among cells exposed to lymphocyte conditioned media plus 1,25(OH)$_2$D$_3$. Vitamin D derivatives with less affinity for 1,25(OH)$_2$D$_3$ receptors[3] were not equivalent to 1,25(OH)$_2$D$_3$. Lymphocyte conditioned media alone acted only as a weak stimulus, and IFN-γ alone or 1,25(OH)$_2$D$_3$ alone had no effect in this system.

These results differ somewhat from reports in which U937 cells' ability to reduce O_2 could be demonstrated after their differentiation with either IFN-γ or 1,25(OH)$_2$D$_3$ alone.[5,8,28-30] However, in these studies different systems for detecting O_2 reduction were used. Also, clones of U937 cells

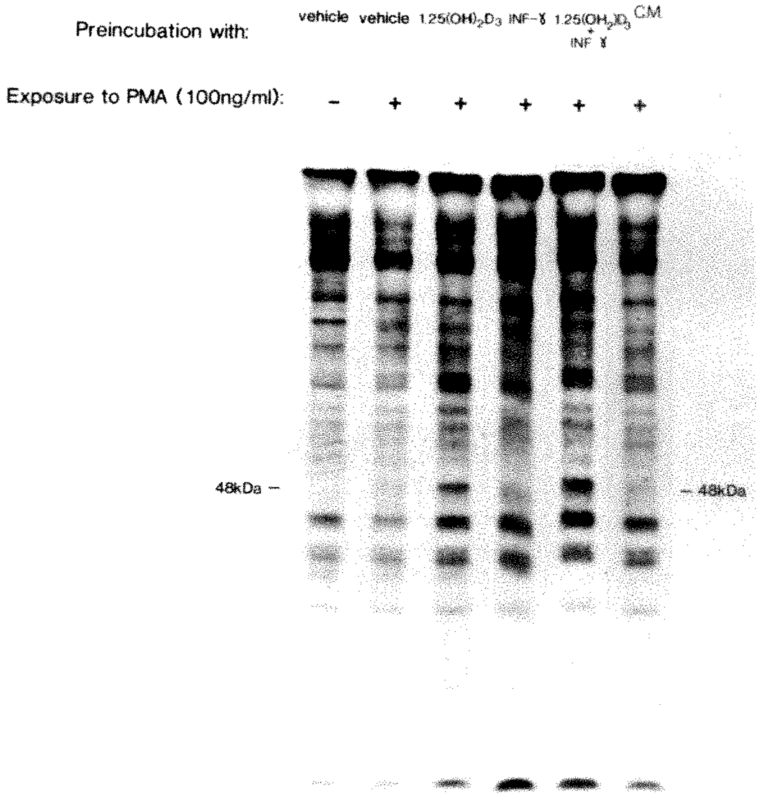

Fig 3. PMA-stimulated phosphorylation in ^{32}P-labeled intact U937 cells. After a six-day exposure to 1,25(OH)$_2$D$_3$ alone or in combination with IFN-γ, IFN-γ, or lymphocyte conditioned media (see Table 3 for specific concentrations), the cells were labeled with ^{32}P for two hours. The labeled cells were exposed to vehicle (−) or 100 ng/mL PMA (+) as shown above the autoradiogram. After ten minutes the reaction was terminated, the substrates were separated by electrophoresis and identified by autoradiography. Location of the 48-kD substrate is indicated on the side of the autoradiogram. This experiment was repeated on three separate occasions with similar results.

clearly differ in their responses to these factors. In earlier work using the same U937 clone as examined in this report we noted a minimal increase in glucose oxidation and no superoxide release among cells differentiated with 1,25(OH)$_2$D$_3$.[4]

Several different mechanisms for expression of LDL by phagocytic cells are possible.[18-21] Because light emission was eliminated by SOD, LDL could primarily be attributed to superoxide anion formation. Myeloperoxidase was present in these cells in equivalent concentration under all conditions examined, so differences in myeloperoxidase formation are unlikely to account for differences in LDL observed.[20]

Formation of superoxide by phagocytic cells involves the transfer of a single electron from NADPH to molecular oxygen.[31] This reaction is mediated by an NADPH-dependent oxidase and a unique cytochrome. PMA-mediated O_2 reduction has been linked to the transfer of protein kinase activity from the cytosol to a particulate fraction[23] and phosphorylation of 43- to 48-kD proteins.[22,25,26] A deficiency in phosphorylation of these substrates has been demonstrated in neutrophils harvested from children with some forms of chronic granulomatous disease of childhood[25,26] where the cells are unable to form superoxide. Increased superoxide formation by activated macrophages has also been related to a reduced K*m* of NADPH oxidase[31,32] and enhanced cytochrome function.[33]

We measured protein kinase C–dependent phosphorylation in U937 cells incubated under conditions known to enhance LDL. The metabolite 1,25(OH)$_2$D$_3$ enhanced maximal protein kinase C–dependent phosphorylation of a 48-kD substrate but did not permit LDL. Lymphocyte conditioned media alone led to LDL but did not enhance 48-kD phosphorylation. Phosphorylation of the 48-kD protein under conditions that allowed maximal LDL, ie, the combination of 1,25(OH)$_2$D$_3$ and lymphocyte conditioned media, was no greater than that of 1,25(OH)$_2$D$_3$ alone. Furthermore, under these conditions LDL but not phosphorylation was greater in adherent cells. These results suggest that although 48-kD phosphorylation may be required for superoxide formation in response to some stimuli the magnitude of phosphorylation cannot be correlated directly with LDL. These results do not preclude the idea that a 1,25(OH)$_2$D$_3$-mediated increase in 48-kD phosphorylation enhances other effects of lymphocyte conditioned media involved in the generation of LDL. Also, it is possible that the conditions of incubation have an effect on translocation of protein kinase C[23] or other critical cytosolic components,[31] and experiments to examine this hypothesis are in progress.

These results are consistent with our recent work showing activation of human monocyte-derived macrophages by 1,25(OH)$_2$D$_3$.[34] In these studies the concentration of 1,25(OH)$_2$D$_3$ required to produce effects was supraphysiological, although serum vitamin D binding proteins reduce the concentration of 1,25(OH)$_2$D$_3$ available to interact with the cell.[35] It seems possible that lower concentrations of 1,25(OH)$_2$D$_3$ are permissive for a variety of cell functions.

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Plasminogen Activator Inhibitor 1: Development of a Radioimmunoassay and Observations on Its Plasma Concentration During Venous Occlusion and After Platelet Aggregation

By Egbert K.O. Kruithof, Grazia Nicolosa, and Fedor Bachmann

To study the effect of plasminogen activator inhibitors (PAI) on fibrinolysis it is essential to be able to specifically measure these proteins in plasma. To this end PAI-1 was purified from cortisol-stimulated HT 1080 fibrosarcoma cells and antisera raised in rabbits. The immunologic relationship of the purified inhibitor to PAI-1 in plasma and platelet extracts was established by immunoblotting and regular and reverse fibrin zymography. Furthermore, the purified product could be immunoprecipitated with antibodies to human or bovine endothelial cell-derived PAI-1. A radioimmunoassay was developed that measures both free and tissue-type plasminogen activator (t-PA)-bound PAI-1 in plasma and has an effective range of 8 to 250 ng/mL. PAI-1 antigen levels showed a twofold increase after 20 minutes of venous occlusion, partially due to hemoconcentration. Approximately one quarter of PAI-1 before and after venous occlusion is derived from platelets. After correction for hemoconcentration and the contribution of platelets to plasma PAI-1 levels, a still significant increase in PAI-1 levels was noted during venous occlusion, which suggests that the local vascular bed releases PAI-1. Concomitant with PAI-1, t-PA antigen levels increased eightfold and fibrinolytic activity 18-fold after 20 minutes of venous occlusion. PAI-1 and t-PA levels tend to augment with age: in a group of older healthy volunteers (mean age, 53 years) PAI-1 levels were twice and t-PA levels 1.7 times higher than those in a group with a mean age of 29 years. Determination of PAI-1 antigen levels before and after platelet aggregation demonstrated that 85% of PAI-1 in platelet-rich plasma is associated with platelets. The average amount of PAI-1 per platelet was 0.3 fg/platelet, ie, 4,000 molecules per platelet.

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THE FIBRINOLYTIC SYSTEM in plasma is a complex enzyme cascade system that degrades fibrin and thereby contributes to the prevention of thrombotic disease. A precise regulation of this system is needed to avoid nonspecific proteolytic degradation of plasma proteins and hemostatic plugs. Plasminogen activator inhibitors (PAIs) are important modulators of fibrinolytic activity.[1-5] Two distinct PAIs have been purified and are well characterized: PAI-1[6,7] and PAI 2.[8-10] PAI-1 is found in normal plasma and platelet releasates,[11-14] whereas PAI-2 is observed in pregnancy plasma[15,16] and in leukocytes.[17-19]

In a variety of clinical conditions increased levels of PAI activity have been observed,[3,15,16,20-24] and it has been suggested that PAI activity behaves as an acute-phase reactant.[25,26] PAI activity assays, however, do not measure the total PAI content of plasma but rather the level of free, active PAI-1. To evaluate correctly the contribution of PAI-1 to fibrinolytic activity it is, therefore, essential to complement PA and PAI activity measurements with specific antigen determinations of tissue-type PA (t-PA) and PAI-1. To this end we have developed a radioimmunoassay (RIA) for PAI-1, applied this assay to the measurement of PAI-1 levels in the plasma of normal individuals before and after venous stasis, and studied the contribution of platelets to PAI-1 levels in plasma.

MATERIALS AND METHODS

Materials. Iodogen was obtained from Pierce, Oud Beijerland, The Netherlands; Na^{125}I from New England Nuclear, Dreieich, FRG; Sephadex G25, Sephadex G100, CNBr-activated Sepharose 4B, concanavalin A (Con A)-Sepharose, and protein A–Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. All reagents for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and the peroxidase staining reagent were obtained from Bio-Rad Laboratories, Richmond, CA. The World Health Organization International Reference Preparation of human t-PA (NIBSC 83/517) was provided by Dr P.J. Gaffney from the National Institute for Biological Standards and Control, London, UK.

t-PA. Human t-PA was purified and antiserum against t-PA raised in rabbits as described.[27] The activity of the t-PA preparation used in this study was calibrated in a fibrin plate assay against that of the international reference preparation.

PAI-1. PAI-1 was purified from the conditioned medium of cortisol (10^{-7} mol/L) stimulated human fibrosarcoma (HT 1080) cells.[7] The cells were grown to confluency in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum in 175-cm^2 culture flasks (Corning Glass Works, Corning, NY), washed twice with phosphate buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.2 g/L KH_2PO_4) and stimulated with 10^{-7} mol/L cortisol (Solu-Cortef, Upjohn, Puurs, Belgium) in DME. Five-liter batches were concentrated 20-fold in a Millipore membrane concentrator equipped with a 4,650-cm^2 Pellikon membrane and passed over a 70 mL Con A–Sepharose column.[6] After washing with 40 mL 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4, and 40 mL 0.05 mol/L Tris-HCl, 1 mol/L NaCl, pH 7.4, PAI-1 was eluted with 0.05 mol/L Tris-HCl, 0.5 mol/L NaCl, 0.5 mol/L methyl-α-mannoside, pH 7.4. The protein-containing fractions of the methylmannoside eluate (approximately 150 mL) were dialyzed against 2 × 4 L of deionized water and, after the addition of 0.1 vol 0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 8.4, passed over a 1.5-mL column of diethyl aminoethyl–Sephacel preequilibrated with 0.01 mol/L Tris-HCl, 0.01 mol/L NaCl, pH 8.4. The column was washed with 10 mL of the same buffer followed by a stepwise gradient of 0.02 to 1 mol/L NaCl in the same buffer. PAI-1, as judged from

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sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), reverse fibrin zymography, and immunoblotting eluted at a NaCl concentration of 0.1 mol/L. The PAI-1–containing fractions (3 mL) were further purified by gel filtration on a 60 × 1.6-cm column of Sephadex G-100 preequilibrated with 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, and 0.0001% merthiolate, pH 7.4. The final product showed one band on reduced and nonreduced SDS-PAGE. The protein concentration of the pure product was determined by the method of Bradford.[28]

PAI-1 was radioiodinated by the iodogen technique.[29] Radioiodinated inhibitor was separated from free ^{125}I by passage over a 10-mL column of Sephadex G-25. Its specific radioactivity was between 15 and 50 nCi/ng (corresponding to 15 to 50 molecules of ^{125}I incorporated per 100 molecules of PAI-1). After radiolabeling, approximately 70% of the inhibitor had retained its ability to bind to anti–PAI-1 antibodies. Under the conditions of the RIA using different antiserum dilutions and the recommended amount of cellulose-bound donkey antibodies to rabbit IgG (Sac-Cel, Wellcome, Beckenham, UK) maximal binding was only 30% at an antiserum dilution of 1:200. This low binding was found to be due to the limited capacity of the Sac-Cel preparation. When immunopurified antibodies and twice the amount of Sac-Cel were used, 70% of the radiolabeled antigen was immunoprecipitated.

Preparation of antiserum to PAI-1. For the immunization of rabbits with PAI-1, the gel-filtered PAI-1 preparation was subjected to further purification by SDS-PAGE in a 10% gel.[30] The stained PAI-1 bands were cut out and mixed by passage through hypodermic needles of different diameter with 0.3 mL 0.2% SDS and 0.5 mL Freund's adjuvant (complete for the first immunization, incomplete for subsequent immunizations).

Total anti–PAI-1 IgG was prepared by passing 1 mL antiserum over 1 mL protein A–Sepharose. After washing with 10 mL 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.5, the IgG was eluted with 0.1 mol/L glycine-HCl, pH 2.2. Immediately after elution the IgG solution was neutralized by addition of 0.1 vol of 1 mol/L Tris. For preparing immobilized anti–PAI-1 IgG, the IgG was dialyzed overnight against two changes of 1 L 0.1 mol/L sodium bicarbonate, pH 8.1, and coupled to 1 mL CNBr-activated Sepharose 4B.[13,16]

Immunopurified anti–PAI-1 antibodies were prepared from total IgG by immunoadsorption to immobilized PAI-1 (150 μg pure PAI-1 coupled to 300 μL CNBr-activated Sepharose) and elution with glycine-HCl as described earlier. Biotinylated anti–PAI-1 IgG was prepared from the immunopurified IgG by incubating for two hours at room temperature 200 μL IgG (0.3 mg/mL in sodium bicarbonate) with 10 μL *N*-hydroxysuccinimidobiotin (Sigma Chemical Co, St Louis; 1 mg/mL in dimethylsulfoxide). Incorporated biotin was separated from free biotin by centrifugation through a 0.6-mL spincolumn of Sephadex G-25. Immobilized immunopurified anti–PAI-1 IgG as well as control IgG was prepared either by direct coupling of 0.5 mg IgG/mL CNBr-activated Sepharose or by incubating for one hour at room temperature 10 μg IgG with 100 μL cellulose-bound donkey antibodies to rabbit IgG (Sac-Cel) followed by removal of the supernatant by centrifugation (five minutes, 1,000 g). For comparative purposes antisera to bovine PAI-1[6] and human PAI-1[31] were obtained from Drs Loskutoff (Scripps Clinic, La Jolla, CA), and Sprengers (Gaubius Institute, Leiden, The Netherlands), respectively.

Plasmas. Blood was obtained by venipuncture from healthy volunteers in the age range of 26 to 59 years before and after ten or 20 minutes of venous occlusion induced by an arm cuff inflated midway between the systolic and diastolic pressures. Women using oral contraceptives were excluded from the study. The blood was collected on ice, anticoagulated with buffered citrate (nine parts of blood were mixed with one part of 0.1 mol/L sodium citrate/citric acid, pH 4.5), and centrifuged for 15 minutes at 1,800 g and 4°C. Plasma was collected and stored at −70°C. Platelet-rich plasma (PRP) was obtained by a ten-minute centrifugation at 200 g and 20°C; the platelet concentration was adjusted to 200 × 10^6 platelets/mL by dilution with the homologous plasma. Platelet aggregation in PRP was induced by collagen (Horm, Hormon Chemie, Munich, FRG; 5 μg/mL final concentration). After a five-minute incubation at 37°C the plasma was centrifuged (one minute, 25°C, 10,000 g) and stored at −70°C as described before.[13] Only those PRPs that exhibited normal aggregation curves in an aggregometer were used in the study. A normal plasma pool was prepared from the blood of 50 healthy blood donors.

An extract of washed platelets was made as described.[13]

Immunoradiometric assay of t-PA in plasma. t-PA antigen levels in human plasma were determined by using an immunoradiometric assay essentially as described by Rijken et al.[32] Standard curves were made by using a t-PA preparation standardized against the international reference preparation of t-PA. The latter has a specific activity of 500,000 IU/mg protein (1 IU = 2 ng).

RIA of PAI-1 in plasma. One hundred microliters of plasma, diluted fivefold with immunodilution buffer (0.05 mol/L Tris-HCl, pH 7.4, 0.2 mol/L NaCl, 0.01 mol/L EDTA, 0.1% bovine serum albumin, and 0.05% NaN_3) was mixed with 100 μL anti–PAI-1 antiserum that had been diluted such (1:1,500) that, in the absence of cold PAI-1, half-maximal binding of the ^{125}I-labeled PAI-1 to the antibodies occurred. After a two-hour incubation at 37°C, 50 μL of ^{125}I–PAI-1 (8,000 cpm) was added and incubated overnight at 4°C. One hundred microliters of a suspension of donkey antirabbit IgG antibodies coupled to cellulose (Sac-Cel) was then added and incubated for 30 minutes at room temperature. Two milliliters of 0.15 mol/L NaCl was added and the mixture centrifuged for five minutes at 200 g. The pellet was washed twice with 2 mL 0.15 mol/L NaCl and counted in a gamma counter. Radioactivities after deduction of nonspecific binding of ^{125}I–PAI-1 to Sac-Cel in the absence of added antiserum (usually less than 30 cpm) were compared in a logit-log plot with a standard curve of PAI-1.

Determination of the fibrinolytic activity of the euglobulin precipitate. After 10 minutes thawing in a 37°C waterbath, 1 vol of plasma was diluted ten times with ice-cold water and acidified by the addition of a 0.75 vol of 0.25% acetic acid (resulting in a pH of approximately 5.9). After 30 minutes at 0°C the preparation was centrifuged for 20 minutes at 1,200 g and 4°C and the pellet redissolved in a 0.5 vol of 0.1 mol/L Tris-HCl, pH 7.5. Fibrinolytic activity of the euglobulin fractions was determined on plasminogen-rich bovine fibrin plates as previously described.[33] For each sample, 30 μL of the euglobulin solution and 5 μL of 17.5 mmol/L sodium flufenamate (Aldrich Chemical Co, Steinheim, FRG) was applied on the fibrin plates.

Electrophoretic-zymographic techniques. Fibrin zymography[34] and reverse fibrin zymography[35] were performed after electrophoretic separation of plasma and platelet proteins by SDS-PAGE in a 10% gel.[30] Immunoblotting[36] was executed as follows: after SDS-PAGE the proteins were electrophoretically (16 hours, 30 V, 100 mA) transferred to nitrocellulose. The membrane was treated with Blotto[37] (5% wt/vol skimmed milk powder in 0.025 mol/L Tris, 0.5 mol/L NaCl, pH 7.5, [TBS]), incubated for three hours at room temperature in 5 mL of biotinylated anti–PAI-1 IgG (2 μg/mL in Blotto), washed three times for 15 minutes with 100 mL Blotto and twice for ten minutes with TBS, incubated one hour at room temperature with a conjugate of horseradish peroxidase with avidin (Vector Laboratories, Inc, Burlingame, CA), washed three times for 15 minutes in TBS, and developed with a peroxidase staining reagent (Bio-Rad).

PAI activity assay. PAI activity in plasma was determined by the indirect chromogenic substrate assay as described by Verheijen et al.[4]

Preparation of PAI-1–deficient plasmas or platelet extracts. Five milliliters of a normal human plasma pool was passed over a 1-mL column of immobilized total anti–PAI-1 IgG (5 to 10 mg of IgG/mL gel) and the protein containing run-through fractions (except for the first milliliter) collected and stored at −70°C. As an alternative, small-scale procedure, PAI-1 or PAI-1/t-PA complexes were removed from plasma or platelet extracts by a one-hour incubation at room temperature with immunopurified or control IgG bound to immobilized antirabbit IgG antibodies (prepared as described earlier). The immunoprecipitant then was removed by centrifugation (five minutes, 1,000 g).

RESULTS

Characterization of the antibodies directed against the PAI purified from HT 1080 cells. HT 1080 cells are able to secrete both PAI-1 and PAI-2.[38] To develop an RIA measuring PAI-1 in human plasma and in platelet extracts it was essential to determine whether the PAI purified by us from corticosteroid-stimulated HT 1080 cells is immunologically related to plasma PAI-1 and whether the antiserum raised reacts with PAI-1 in plasma and platelet extracts. To establish the specificity of the antiserum, we subjected an extract of washed platelets to SDS-PAGE followed by reverse fibrin autography as well as immunoblotting using biotinylated anti–PAI-1 IgG. A 51-kd immunoreactive band was observed in immunoblotting (Fig 1, lane 1). This band comigrated with the lysis inhibition band seen in reverse fibrin autography; the latter band could be removed by immunoprecipitation of the platelet extract with immunopurified anti–PAI-1 antibodies (Fig 1, lanes 2 and 3).

The addition of an excess of t-PA (5 U/mL, final concentration) to human plasma resulted in the formation of a 110-kDa t-PA/PAI-1 complex band; a minor amount of t-PA remained in its free form. Immunoprecipitation using immunopurified anti–PAI-1 antibodies completely removed the 110 kDa band, whereas the 67-kDa t-PA band still remained visible, thus demonstrating that the anti–PAI-1 antibodies also recognize PAI-1 complexed to t-PA (Fig 1).

Further proof of the immunologic relationship of the PAI purified by us from HT 1080 cells with PAI-1 was obtained by immunoprecipitation of radiolabeled PAI with antisera to bovine or human endothelial cell–derived PAI-1 (Fig 2). With our antiserum, half-maximal binding occurred at an antiserum dilution of 1:1,500. When using the antisera against PAI-1 from human or bovine endothelial cells, half-maximal binding occurred at lesser dilutions, but the slopes of the three curves was comparable. Preincubation of our antiserum with different concentrations of the PAI purified by us from HT 1080 cell conditioned medium or with a PAI-1 preparation that, by aminoacid sequence analysis, was identical to the endothelial cell–derived PAI-1[39] (a kind gift of Dr Andreasen) resulted in similar PAI concentration-dependent binding inhibition curves. Thus, the specificity and utility of these antigen and antisera preparations for the development of an RIA for measuring PAI-1 has been established.

Determination of optimal plasma dilutions for the assay of PAI-1. The presence of a fairly high concentration of proteins in plasma may influence the binding of an antigen to antibodies. To validly use standard curves for PAI-1 in immunodilution buffer it was therefore essential to examine

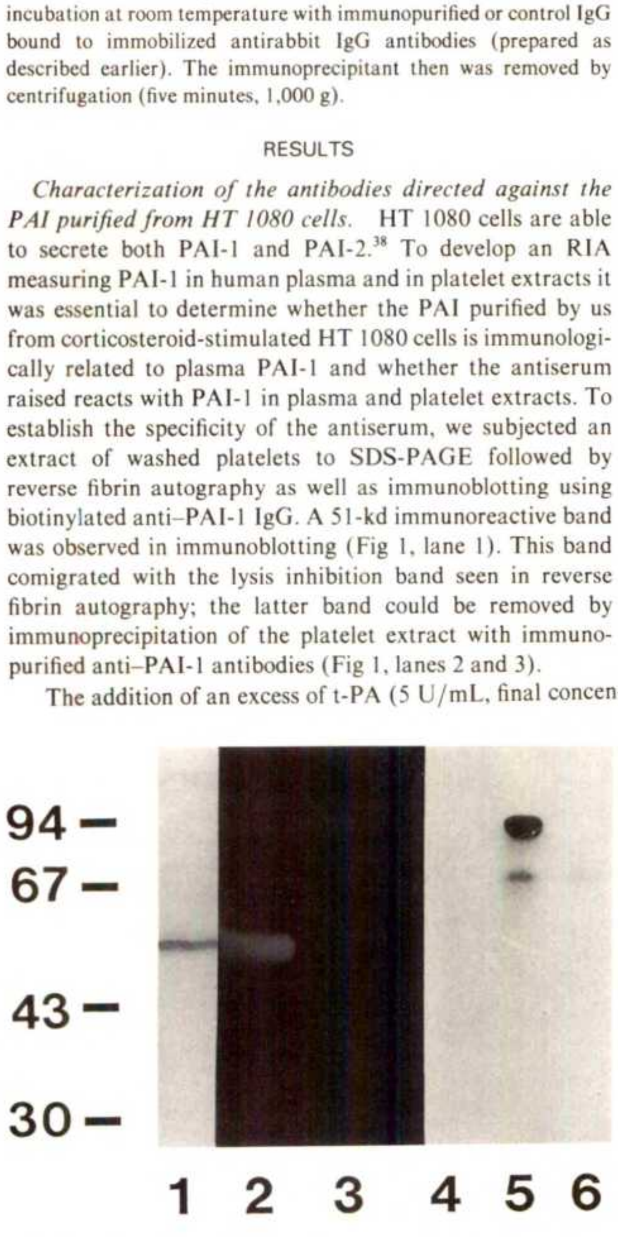

Fig 1. Analysis of the specificity of the anti–PAI-1 antiserum. Lane 1, extract of washed platelets subjected to SDS-PAGE and immunoblotting using biotinylated anti–PAI-1 IgG; lanes 2 and 3, extracts of washed platelets subjected to SDS-PAGE followed by reverse fibrin zymography; lanes 4 to 6, regular fibrin zymography of euglobulin precipitates prepared from human plasma before (lane 4) or after (lanes 5 and 6) the addition of 5 U/mL t-PA. Lane 3 and 6 show a platelet extract or plasma euglobulins, respectively, after immunodepletion with immunopurified anti–PAI-1 IgG. The positions of the molecular weight (mol wt) standard proteins phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carboanhydrase (30,000) are indicated at the left.

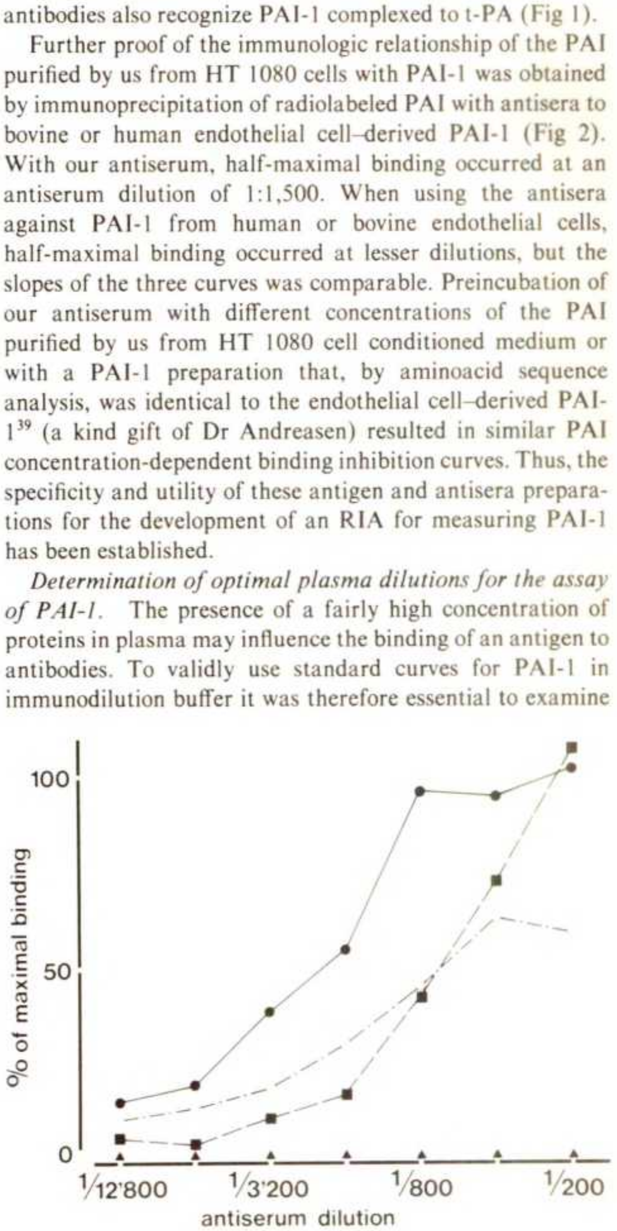

Fig 2. Binding of the PAI purified from HT 1080 cells to antibodies to human and bovine PAI-1. Radioiodinated PAI-1 (0.2 ng, 8,000 cpm) was incubated overnight with various dilutions of antiserum prepared against PAI purified from HT 1080 cells (●), PAI-1 purified from human (■) or bovine (○) endothelial cells, or with preimmune rabbit antiserum (▲). The complexes of ^{125}I-PAI with rabbit antibodies were precipitated with cellulose-bound donkey antibodies to rabbit IgG (see Materials and Methods) and, after washing the cellulose, counted in a gamma counter. The results are expressed as percentages of maximal binding, which, under the conditions of the RIA, was 30% at a 200-fold dilution of antiserum against human PAI-1. By using immunopurified IgG maximal binding was approximately 70%.

whether, at the plasma dilutions used, the binding of antigen is affected. To this end standard curves of PAI-1 in buffer and in different dilutions of PAI-1–depleted normal plasma were made by using the antiserum dilution (1:1,500) that resulted in half-maximal binding of labeled PAI-1 in the absence of added cold antigen. Standard curves of PAI-1 in plasma diluted five times or more were essentially identical to those made in immunodilution buffer (Fig 3). Furthermore, the dilution curve of pure PAI-1 added to plasma was parallel to that of plasma containing endogenous PAI-1. Henceforth, fivefold-diluted plasma was used for all PAI-1 determinations. Figure 3 shows that the range of the RIA for PAI-1 was 1.6 to 50 ng/mL. This results in a factual range in plasma of 8 to 250 ng/mL and allows a direct assay of PAI-1 antigen in over 95% of all plasma samples.

Correlation of PAI activity and PAI-1 antigen. In the plasma of 39 healthy individuals (19 males, 20 females) there was a significant correlation (Spearman's rank correlation coefficient, .613; $P < .001$) between PAI-1 antigen measured by RIA and PAI activity (Fig 4). However, a threefold discrepancy between antigen and activity was noted. A concentration of 50 ng/mL active PAI-1 corresponds to approximately 30 U/mL (based upon a specific activity of t-PA of 0.5 U/ng and the respective mol wts of t-PA and PAI-1. Instead, the average PAI activity of plasma samples containing a mean PAI-1 concentration of 50 ng/mL was only 9 U/mL.

Intraassay and interassay variability of the RIA. To establish the intraasay variability of the RIA, four sets of 18 duplicate plasma samples were randomly distributed into four assay series and the RIA performed the same day. Interassay variability was determined by testing the same plasma samples on three different days. The intraassay variability was dependent on the PAI-1 concentration. At low concentrations (between 10 and 20 ng/mL) the average intraassay variability (SD/mean value × 100%) was 15%, whereas above 20 ng/mL it was 7%. The average interassay variability was approximately 10%. Because the majority of plasmas show PAI-1 concentrations above 20 ng/mL (see the following sections), the RIA permits the measurement of PAI-1 in clinical conditions with increased PAI-1 levels with a fairly good accuracy.

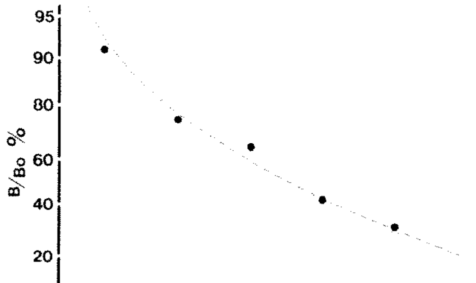

Fig 3. Comparison of standard curves of PAI-1 in fivefold-diluted, PAI-1–depleted plasma and in immunodilution buffer. Different concentrations of PAI-1 in either immunodilution buffer (O) or fivefold-diluted, PAI-1–depleted plasma (●) were incubated for two hours at 37°C with 1,500-fold-depleted antiserum to human PAI-1. Thereafter radiolabeled PAI-1 (0.2 ng, 8,000 cpm) was added and incubated overnight. Bound PAI-1 was separated from unbound by immunoprecipitation of the complexes with immobilized donkey antibodies to rabbit IgG (see Materials and Methods) and, after washing, counted in a gamma counter. The figure shows a logit-log plot of percent bound radioactivity *v* added unlabeled PAI-1.

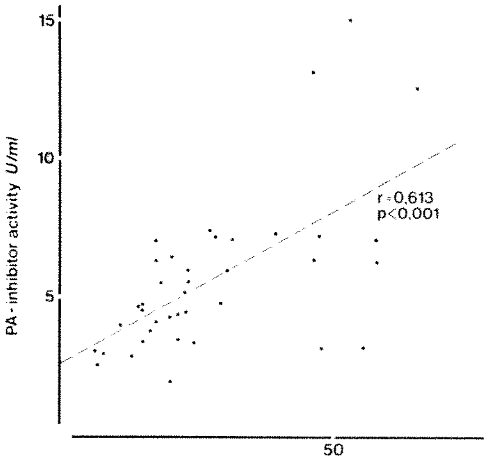

Fig 4. Correlation between PAI-1 antigen levels and PAI activity in normal human plasma. PAI-1 antigen levels and PAI activity were measured in the plasma of 39 healthy volunteers aged 22 to 58.

Effect of complexation of PAI-1 to t-PA on the measurement of PAI-1 antigen. Because PAI-1 exists in human plasma in a free and a t-PA–bound form, it was important to establish to what degree the RIA measures t-PA–complexed inhibitor. To this end PAI-1 antigen determinations were performed in ten normal plasmas before and after the addition of 10 U/mL exogenous t-PA. PAI-1 values in plasmas with or without added t-PA were not significantly different ($P > .2$; Wilcoxon signed rank test) and in general within the intraassay variability. The RIA thus effectively measures both free and t-PA–bound PAI-1.

Influence of sex and age on PAI-1 antigen levels. No significant difference was observed in plasma PAI-1 antigen levels in males (mean ± SD, 31 ± 18 ng/mL; median, 23 ng/mL; range, 6 to 65 ng/mL) and females (mean ± SD, 23 ± 12 ng/mL; median, 21 ng/mL; range, 6 to 57 ng/mL).

A significant correlation of PAI-1 antigen levels with age was observed ($r = .474$, $P < .005$). Dividing the 39 blood donors into three equal groups based upon their age indicated that PAI-1 antigen levels were twofold higher in the oldest than in the youngest group (Table 1). The difference in PAI activity was less marked: the average PAI activity was 40% higher in the oldest age group ($P > .05$). The lesser difference in PAI activity between the two age groups probably is due to the 70% higher t-PA level in the oldest group (Table 1), which leads to a proportionately lower fraction of free PAI-1 in the older individuals.

Release of PAI-1 by human platelets. Collagen stimulation of ten PRPs adjusted to 200×10^6 platelets/mL resulted

Table 1. Effect of Age on PAI-1 and t-PA Antigens and PAI Activity

Group	Age (yr)	PAI-1 (ng/mL)	t-PA (ng/mL)	PAI Activity (U/mL)
1	29 ± 3*	18 ± 6	2.9 ± 0.9	4.8 ± 1.7
2	40 ± 3	26 ± 15	3.4 ± 1.7	5.1 ± 2.7
3	53 ± 4	38 ± 18	4.8 ± 2.4	6.7 ± 3.5
P† of 1 v 3		<.002	<.02	NS

Plasma antigen concentrations of PAI-1 and t-PA as well as PAI activity was determined in the plasma of 39 healthy volunteers. The collective was divided into three groups according to age.

*Mean ± SD of 13 individuals.

†P values were determined with the Mann-Whitney rank correlation test.

NS: $P > 0.05$

in a significant increase of PAI-1 antigen levels ($P < .002$; Wilcoxon signed rank test) from 22 ± 13 ng/mL (mean ± SD, median, 21 ng/mL; range, 8 to 45 ng/mL) in platelet-poor plasma (PPP) to 79 ± 30 ng/mL (median, 78 ng/mL; range, 40 to 137) in the PRPs after platelet aggregation. From the increase in PAI-1 antigen levels after platelet aggregation (57 ng/200 × 10^6 platelets) the amount of platelet-associated PAI-1 was calculated to be 0.3 fg/platelet (or approximately 4,000 molecules of PAI-1/platelet). No correlation ($r = .05$) was observed between plasma PAI-1 levels and platelet-associated PAI-1, which indicated that platelets and plasma constitute two independent compartments for PAI-1. The mean, total platelet-associated PAI-1 calculated from the amount of PAI-1 released by 200 × 10^6 platelets and corrected for each individual platelet count was 139 ± 55 ng/mL, a value sixfold higher than that of PPP.

PAI-1 and t-PA antigen concentrations before and after venous occlusion. During venous occlusion plasma antigen concentrations of both PAI-1 and t-PA antigen augmented. PAI-1 antigen levels increased from 27 ± 16 ng/mL (median, 22 ng/mL) to 43 ± 33 ng/mL (median, 38 ng/mL) after ten minutes and to 59 ± 53 ng/mL (median, 44 ng/mL) after 20 minutes of venous occlusion (Fig 5). Concomitantly, t-PA antigen levels increased from 3.4 ng/mL to 12 and 26 ng/mL after ten and 20 minutes, respectively, of venous occlusion (Fig 5). Several mechanisms may be responsible for the increase in PAI-1 concentrations: (*a*) hemoconcentration induced by the venous occlusion, (*b*) release of PAI-1 by partially activated platelets, and (*c*) release of PAI-1 by local sources, eg, endothelial cells. To evaluate the effect of hemoconcentration, the total protein level of each individual plasma was determined; mean plasma protein concentrations increased 27% after ten minutes and 38% ($P < .001$, Wilcoxon signed rank test; Table 2) after 20 minutes of venous occlusion. After correction of each PAI-1 value for the corresponding hemoconcentration, PAI-1 concentrations after a 20-minute venostasis still were significantly ($P < .001$) increased with respect to those before stasis. To assess the contribution of platelets to stasis-induced augmentations of PAI-1, the extent of the platelet release reaction was estimated from β-thromboglobulin (β-TG) levels. Concentrations of β-TG increased significantly from 320 to 581 ng/mL, but after correction for hemoconcentration this increase was not significant anymore. Because platelets release β-TG and PAI-1 simultaneously,[11,13] it is obvious that part of the PAI-1 in the plasma before and after venous occlusion stems from platelets. Linear regression analysis of PAI-1 and β-TG levels before venous occlusion yielded a regression coefficient of .312 (not significant). The increases in PAI-1 and β-TG levels after correction for hemoconcentration were correlated in a borderline manner ($r = .333$, $P = .04$). From the slopes of the latter regression curve, it was estimated that an increase of 100 ng/mL of β-TG corresponds to an increase of 2.2 ng/mL of PAI-1 (ratio of PAI-1/β-TG, 0.022). These values are slightly higher than those arrived at by determining β-TG and PAI-1 concentrations in PRP before and after induction of platelet aggregation (β-TG, 12 μg/mL; PAI-1, 140 ng/mL; PAI-1/β-TG ratio, 0.012; average determination for ten plasmas). To avoid underestimating the contribution of platelets to increases in PAI-1 concentrations before and after venous occlusion, we have chosen the larger of the two ratios for our calculations.

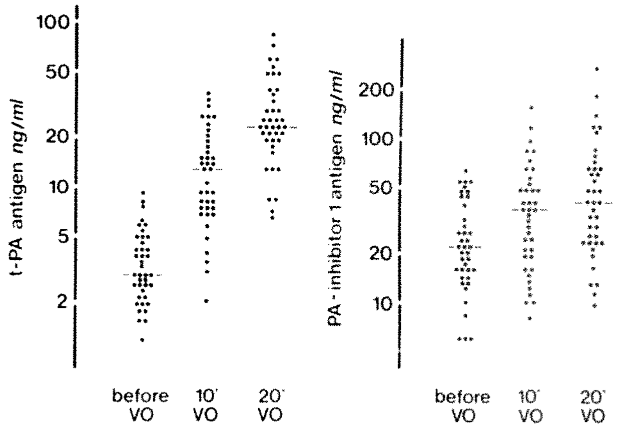

Fig 5. Effect of venous occlusion (VO) on PAI-1 and t-PA antigen levels in normal volunteers. PAI-1 and t-PA antigen levels were measured in the plasma of 39 healthy volunteers before and after ten and 20 minutes of VO.

Table 2 shows that both before and after venous occlusion approximately 25% of PAI-1 antigen was derived from platelets. After correction for the contribution of platelets and hemostasis a significant increase in PAI-1 levels still occurred after ten and 20 minutes of venous occlusion ($P < .02$ and $P < .001$, respectively, Wilcoxon signed rank test).

The effect of the increases in both t-PA and its inhibitor on the fibrinolytic activity of the euglobulins was measured on fibrin plates. It increased from an average of 0.3 U/mL before to 1.7 and 5.4 U/mL after ten and 20 minutes of venous occlusion, respectively. Before venous occlusion fibrinolytic activity was not correlated to t-PA or to PAI-1 levels (Table 3). After ten minutes of venous occlusion, fibrinolytic activity was positively correlated with t-PA levels and negatively with PAI-1 levels. After 20 minutes of venous occlusion, fibrinolytic activity was even more strongly correlated with t-PA levels but no longer with PAI-1 levels. Interesting-

Table 2. Effect of Hemoconcentration and Platelet Activation on Plasma Antigen Concentration of PAI-1

	pre-VO	10-min VO	20-min VO
Hemoconcentration	1.00	1.27 ± 0.15	1.38 ± 0.18
PAI-1	27 ± 16	43 ± 33*	59 ± 53*
	(6-58; 22)	(8-160; 38)	(10-275; 44)
PAI-1 (C)	27 ± 16	34 ± 25*	40 ± 36*
	(6-58; 22)	(7-119; 28)	(8-202; 33)
β-TG	320 ± 215	467 ± 356	581 ± 442
	(80-1,020; 260)	(100-2,000; 360)	(120-2,000; 470)
β-TG (C)	320 ± 215	370 ± 270†	420 ± 300†
	(80-1,020; 260)	(90-1,300; 290)	(90-1,300; 300)
Plasma PAI-1 (C)	20 ± 15	26 ± 23‡	31 ± 35*
	(0-61; 15)	(0-110; 21)	(0-189; 23)
Platelet PAI-1 (C)	7 ± 5	8 ± 6	9 ± 6
	(2-22; 6)	(2-35; 7)	(2-29; 7)

Plasma concentrations of PAI-1 and β-TG are given before and after correction (C) for hemoconcentration. The contribution of platelets to PAI-1 (C) concentrations in each plasma was estimated from β-TG (C) concentrations in that plasma, by assuming a PAI-1/β-TG ratio of 0.022. All protein concentrations are given in nanograms per milliliter. *P* values are given with respect to pre-VO concentrations. Results are given as means ± SD; between parentheses are given range and median values.

Abbreviation: VO, venous occlusion.

**P* < .001.

†*P* > .05.

‡*P* < .02.

ly, before venous occlusion a striking positive correlation was observed between t-PA antigen levels and both PAI activity and PAI-1 antigen levels (Table 3).

DISCUSSION

Only recently was the key role of PAIs on fibrinolytic activity in human plasma established.[1-5] A number of assay systems have been developed that estimate PAI activity in human plasma.[1-4] These assay systems were based on the inhibition of t-PA and measure PAI levels only indirectly. They are sensitive to interference by other, nonspecific inhibitors of t-PA[2] and do not distinguish between different PAIs. Furthermore, stimuli such as exercise that induce the release of t-PA reduce PAI activity. Assays can be rendered more specific for PAI-1 by using antibodies to PAI-1 in conjunction with a PAI activity determination.[40,41] To better understand the contribution of PAI-1 to overall activity of the fibrinolytic system we have developed a PAI-1 antigen assay.

Table 3. Correlation Between PAI-1 Antigen, PAI Activity, t-PA Antigen, and Fibrinolytic Activity Before and After Ten and 20 Minutes of Venous Occlusion

Correlation	Before VO	10-min VO	20-min VO
FA *v* t-PA antigen	.037	.457*	.671*
FA *v* PAI-1 antigen	−.176	−.329†	−.059
PAI activity *v* t-PA antigen	.479‡	ND	ND
PAI activity *v* PAI-1 antigen	.613*	ND	ND
t-PA antigen *v* PAI-1 antigen	.602*	ND	ND

The table gives Spearman's rank correlation coefficients.

Abbreviations: FA, fibrinolytic activity; ND, not done.

**P* < .001.

†*P* < .05.

‡*P* < .005.

We have used cortisol-stimulated HT 1080 fibrosarcoma cells as a source of PAI-1.[7] Because these cells are able to secrete both PAI-1 and PAI-2,[38] it was essential to establish that the purified PAI indeed corresponds to PAI-1. Its immunologic relationship with PAI-1 and the specificity of the antiserum raised against this protein were established by the following criteria: (*a*) in platelet extracts one 51-kDa immunoreactive band comigrated with the platelet PAI-1 band, which was demonstrable by reverse fibrin autography[11,13,14]; the latter was immunoprecipitable with antibodies raised against the HT 1080 PAI. (*b*) The addition of t-PA to human plasma resulted in the formation of a 110-kDa complex with PAI-1.[2,5,13] The complex but not free t-PA reacted with our immunopurified antibodies. (*c*) Radiolabeled HT 1080–derived PAI was immunoprecipitated by antibodies to human or bovine endothelial cell–derived PAI-1. (*d*) A PAI-1 preparation that by criteria of amino acid sequence analysis is identical to PAI-1 (a gift of Dr Andreasen[39]) showed a concentration-dependent inhibition curve of the binding of radiolabeled PAI to our antibodies. These results thus are in agreement with those of others[7,39,42] who purified PAI-1 from HT 1080 cells by a slightly different technique.

Standard curves of PAI-1 in PAI-1–depleted plasma were identical to those in immunodilution buffer, provided the plasma had been diluted at least fivefold. At this plasma dilution, the RIA has a range of 8 to 250 ng/mL and allows, therefore, the direct determination of PAI-1 levels in resting (range, 6 to 57 ng/mL) and postocclusion plasma (10 to 275 ng/mL) (Fig 5). No difference was observed between free and t-PA–bound PAI-1. The assay thus measures the total PAI-1 concentration.

A threefold discrepancy between PAI-1 antigen and PAI activity was noted. Part of this discrepancy is due to the measurement of complexes of PAI-1 with t-PA that are obviously inactive. A further explanation for the lower PAI activity levels may be ascribed to the presence, in plasma, of an inactive, "latent" form of PAI-1 that can be (re-)activated by chaotropic agents.[31,43] In endothelial cell conditioned medium, the active form is converted into the latent form with a half-life of a few hours,[44] and after four hours of culture the ratio of latent to active PAI-1 was more than 12. In human serum a ratio of over 3 was observed.[45] Because in these studies the completeness of activation of the latent form of PAI-1 was not established, actual ratios of latent to active PAI-1 may even have been higher.

As has been observed before for PAI activity in plasma,[1-4] PAI-1 antigen levels likewise were extremely variable: in normal individuals there was a more than tenfold difference between the lowest and highest values. PAI-1 antigen levels were the same in males and females but increased with age. Average PAI-1 antigen levels were twofold higher in the oldest group of volunteers, aged 53 ± 4 years, as compared with the youngest group, aged 29 ± 3 years. This twofold increase did not match entirely a nonsignificant 40% increase in PAI activity. The lesser increase in PAI activity than t-PA antigen concentration may be due, at least in part, to an increased t-PA level in the higher age groups. Our results are in accordance with those of Aillaud et al[46] who recently found that PAI activity and t-PA antigen levels were 60% and 120% higher, respectively, in a group of elderly individuals (average age of 80 years) as compared with a young control group. Whether the increases in PAI-1 and t-PA levels are truly an age-related phenomen or merely reflect an increased occurrence of subclinical pathologies at a higher age remains to be established.

During venous occlusion, antigen levels of both t-PA and PAI-1 increased linearly with time. After 20 minutes of venous occlusion PAI-1 levels had increased twofold and t-PA levels eightfold. Correction for hemoconcentration according to total plasma protein concentrations indicated that half of the increase in PAI-1 levels was due to hemoconcentration. Measurement of β-TG in these plasmas suggested that both before and after venous occlusion approximately one quarter of PAI-1 was derived from platelets that had been partially activated. After correction for platelet-derived PAI-1, a significant increase in PAI-1 was still observed after 20 minutes of venous occlusion. This suggests that the local vascular bed contributes to PAI-1 levels, most likely by release of PAI-1 from endothelial cells.[6,31,43] The corrected increases in t-PA levels (fivefold) are much greater than those of PAI-1 (50%), thereby resulting in general in a net increase in fibrinolytic activity.

In resting plasma no correlation was observed between t-PA or PAI-1 levels and fibrinolytic activity as measured in twofold-concentrated euglobulins. Apparently, in resting plasma the larger part of t-PA exists in an inactive form, and "resting" fibrinolytic activity is little influenced by PAI-1. After ten minutes of occlusion, the average PAI-1 levels were 43 ng/mL, and average t-PA levels were 12 ng/mL. Assuming that only one third of PAI-1 is in an active form, the average concentrations of active PAI-1 and t-PA are roughly equimolar. As expected in such a situation, a positive correlation of fibrinolytic activity with t-PA levels and a negative one with PAI-1 levels was observed. This suggests that, after ten minutes of venous occlusion, fibrinolytic activity reflects a balance between activator and inhibitor and implies that a deficient fibrinolytic activity may be due to a high concentration of PAI-1 or a deficient release of t-PA. At longer occlusion times (20 minutes), the amount of t-PA secreted is in such an excess over active PAI-1 that fluctuations of PAI-1 contribute much less to fluctuations of fibrinolytic activity.

In the resting plasma of healthy individuals a striking correlation was observed between t-PA antigen concentrations on the one hand and PAI-1 antigen levels and PAI activity on the other. This correlation could be explained if the t-PA/PAI-1 complexes had a longer in vivo half-life than free PAI-1 or t-PA or if there was a coordinate regulation of the release and/or clearance of PAI-1 and t-PA. In rabbits, the half-life of complexes of t-PA with PAI-1 and of free t-PA was only a few minutes[47] and shorter than that those of PAI activity (seven minutes).[22] It therefore seems more likely that a common mechanism regulates plasma levels of PAI-1 and t-PA.

Direct measurement of PAI-1 antigen levels before and after platelet aggregation confirms previous results of activity assays and immunologic studies that platelets release PAI-1.[11,13,14] The amount of PAI-1 per platelet (0.3 fg or 4,000 molecules) is quite small. Nevertheless, quantitation of PAI-1 released by platelets suggest that in human blood the larger part of PAI-1 (approximately 85%) is associated with platelets, as has been estimated previously from PAI activity assays.[13,14] In the present study in which plasma was prepared as recommended for fibrinolytic tests, approximately one quarter of PAI-1 was derived from platelets. The lack of correlation, however, between plasma and platelet-associated PAI-1 concentrations and the poor correlation between β-TG and PAI-1 levels in resting and poststasis plasma confirms previous suggestions that plasma and platelets constitute two independent compartments of PAI-1.[13,14] However, the presence of β-TG in the citrated plasmas indicated that approximately 25% of PAI-1 in plasma actually was derived from platelets. By collecting the blood on anticoagulants that prevent platelet release, eg, those recommended for measuring β-TG in plasma, a more precise determination of the plasma concentration of PAI-1 could be achieved.

The development of a specific RIA for PAI-1 now permits studies on the fluctuations of PAI-1 antigen in health and disease. As exemplified by the modifications of PAI-1 antigen and PAI activity with age, antigen measurements may be more sensitive in detecting increases in PAI-1 levels than are activity assays.

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Reciprocal Translocation Involving the Short Arms of Chromosomes 7 and 11, t(7p-;11p+), Associated With Myeloid Leukemia With Maturation

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A reciprocal translocation involving the short arms of chromosomes 7 and 11, t(7;11)(p15;p15), was found in nine patients including eight with acute myelogenous leukemia (AML) and one with Philadelphia (Ph^1) chromosome-positive chronic myelogenous leukemia (CML) in blastic crisis. Although a similar chromosome rearrangement has previously been reported in five patients, including three with AML and two with CML, the 7p breakpoint in some of these cases was slightly different from that detected in our patients. Notable cytogenetic and clinicohematologic findings in our patients and those reported in the literature were as follows: (*a*) t(7;11) occurred in myeloid leukemia, predominantly AML with subtype M2, and occasionally in other AML subtypes and in CML with or without Ph^1 chromosome; (*b*) t(7;11) frequently occurred as the sole chromosome abnormality; (*c*) most patients showed a low neutrophil alkaline phosphatase score; and (*d*) Auer rods were present in leukemic cells of most cases including Ph^1-positive CML. Our findings suggest that a t(7;11)-associated leukemia constitutes a subgroup of myeloid malignancy involving maturing leukemic cells.

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IT IS NOW WELL ESTABLISHED that specific chromosome abnormalities correlate with a particular type of leukemia showing characteristic morphological and clinical features. The best known examples are Philadelphia (Ph^1) translocation, t(9;22), in chronic myelogenous leukemia (CML)[1] and t(8;21), t(15;17), and inv(16) in acute myelogenous leukemia (AML) with subtypes M2, M3, and M4 with abnormal eosinophils, respectively.[2-7] These specific chromosome abnormalities have a diaganostic and sometimes a prognostic significance. Recently, a reciprocal translocation involving the short arms of chromosomes 7 and 11, t(7p−;11p+), was reported in several Japanese patients with leukemia.[8-12] Although a possible association of t(7;11) with Ph^1-negative CML has been hypothesized,[11] the clinicohematologic findings thus far obtained from the patients with this abnormality seem to be insufficient to warrant this association.

In the present investigation, we studied nine patients with t(7;11), including eight with AML and one with Ph^1-positive CML, and paid particular attention to their clinicohematologic characteristics.

MATERIALS AND METHODS

Patients. Nine patients including eight with AML and one with Ph^1-positive CML in blastic crisis were evaluated and treated at the following Japanese institutes; Asahikawa Red Cross Hospital, Hokkaido University Hospital, Jichi Medical School Hospital, Nihon University Itabashi Hospital, Kinki Central Hospital, Hiroshima University Hospital, Medical College of Oita Hospital, and Naha Prefectural Hospital. Informed consent for chemotherapeutic procedures and necessary studies was obtained from all the patients. Diagnosis and classification of leukemias were based on morphological and cytochemical examinations of peripheral blood smears and bone marrow aspirates on admission. The eight AML patients included seven with subtype M2 and one with M5b according to classification by the French-American-British Cooperative Group criteria.[13] Neutrophil alkaline phosphatase (NAP) score was counted according to the method described by Tomonaga et al.[14] All eight AML patients were given intensive combination chemotherapy with cytosine arabinoside (Ara-C) or N^4-behenoyl-1-β-D-arabinofuranosylcytosine (BH-AC; Asahi Chemical Industry, Osaka, Japan), daunorubicin (DNR) or aclarubicin hydrochloride (ACM; Aclacinomycin A, Yamanouchi Pharmaceutical Co, Ltd, Tokyo), 6-mercaptopurine (6-MP), and prednisolone (PSL). In some cases, either interferon-α (INF-α) or vincristine (VCR) was added as initial induction chemotherapy. The CML patient (patient no. 9) was treated with busulfan in the chronic phase and with intensive combination chemotherapy with BH-AC, DNR, VCR, and PSL in the blastic phase.

Cytogenetic study. Chromosome analysis with Q- or G-banding techniques was performed on bone marrow and peripheral blood cells obtained from each patient at the time of diagnosis. Patient nos. 1 and 5 were examined two or three times during the clinical course. One Ph^1-positive CML patient (patient no. 9) was examined both in the chronic and blastic phases. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[15]

RESULTS

Hematologic and clinical findings. The hematologic and clinical findings in the nine patients examined at the time of diagnosis or at the onset of blastic crisis are summarized in Table 1. The ages of these patients, who included five males and four females, ranged from 16 to 59 years old (median, 40). None of the patients except for the CML

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Table 1. Clinical, Hematologic, and Cytochemical Data of the Patients With t(7;11)

			Peripheral Blood					Bone Marrow										
Case No.	Age/Sex	Diagnosis	WBC (× 10^9/L)	Blasts (%)	Hb (g/dL)	Platelet (× 10^9/L)	NAP* Score	NCC (× 10^9/L)	Blasts (%)	Prom (%)	Mature Granulo (%)	Auer Rods	POX	AS-D ch	α-NB	Therapy	RM	Survival† (mo)
1	36/F	AML(M2)	48.5	12	5.9	3.6	33	66.0	42.0	17.6	23.8	+	+	–	–	A	Yes	31
2	16/M	AML(M2)	69.7	91	11.7	9.6	ND	12.5	78.4	5.2	0.2	+	+	–	–	B	No	6
3	38/F	AML(M2)	3.2	5	8.0	12.7	13	10.0	66.1	2.1	14.5	+	+	+	–	INF-α, B	Yes	27
4	40/F	AML(M2)	3.29	13	10.0	2.9	dec	16.7	52.2	8.4	7.4	+	+	–	–	B	Yes	5
5	42/M	AML(M2)	35.2	22	10.5	12.1	dec	87.0	40.8	9.0	32.0	+	+	ND	–	B, VCR	Yes	13
6	51/M	AML(M2)	8.2	45	7.7	5.1	82	15.0	54.6	4.5	19.5	+	+	–	–	B	Yes	26+
7	51/M	AML(M2)	2.4	3	9.3	3.0	95	4.8	37.0	7.9	10.8	+	+	–	–	B	Yes	13
8	28/M	AML(M5b)	54.2	20	8.6	7.2	ND	Hyper	13.6	–‡	5.2	+	+	ND	+	B	Yes	17
9	59/F	Ph^1 (+) CML-BC	51.2	8	10.4	54.4	51	Hyper	57.4	19.0	3.0	+	+	+	–	C	No	58 (4)

Abbreviations: NCC, nucleated cell count; Prom, promyelocytes; Granulo, granulocytes; POX, peroxidase; AS-Dch, napthol-AS-D-chloroacetate esterase; α-NB, α-naphthyl butyrnate esterase; RM, remission; A, BH-AC, ACM, 6-MP, and PSL; ND, not done; B, BH-AC, DNR, 6-MP, and PSL; dec, deceased; Hyper, hypercellularity; C, BH-AC, DNR, VCR, and PSL; BC, blastic crisis.

*Tomonaga's method; normal value, 177 to 350.

†The number in parenthesis shows the survival time from blastic crisis.

‡In this patient, the percentage of monocytoid elements was 48.7%. See the text for the detail.

patient (patient no. 9) had a previous history of chemotherapy or mutagen exposure. Organomegaly was found in three patients, one (patient no. 6) with hepatosplenomegaly and two (patient nos. 3 and 9) with hepatomegaly.

In peripheral blood, the WBC count ranged from 2.4 to 69.7 × 10^9/L (median, 35.2 × 10^9/L), the hemoglobin level from 5.9 to 11.7 g/dL (median 9.3 g/dL), and the platelet count from 2.9 to 54.4 × 10^9/L (median, 7.2 × 10^9/L). The blast count ranged from 3% to 91% (median, 13%). Bone marrow aspirates showed a various number of myeloblasts, promyelocytes, and maturing granulocytic elements (Fig 1A). The proportion of myeloblasts in patient nos. 1 to 7 ranged from 37.0% to 78.4%. In patient no. 8, the proportion of myeloblasts was 13.6%, but that of monocytoid elements showing α-naphthyl butyrate esterase activity with NaF inhibition was 48.7%. In all patients, the myeloblasts exhibited peroxidase activity (Fig 1B), and in two, a fraction of myeloblasts showed naphthol-AS-D-chloroacetate esterase activity (patient nos. 3 and 9). No eosinophilia was observed in the bone marrow (below 2.8%) or the peripheral blood (below 1.0%) of all patients, although abnormal eosinophils with basophilic granules were found in two patient (patient nos. 1 and 2). Large myelocytes and metamyelocytes, and giant mature polymorphs were found in some patients (patient nos. 1 to 4). Auer rods were found in the cytoplasm of a fraction of the blasts from all patients including the patient with Ph^1-positive CML (patient no. 9); they were usually single and occasionally two or three in a leukemic blast (Fig 1C), but were not in mature polymorphs of any patient. NAP score (normal value, 177 to 350) was decreased in seven of the patients, including the CML patient in blastic crisis. Patient no. 6 was diagnosed as having M2 when first admitted, but at relapse 13 months later, his leukemic blasts exhibited myelomonocytoid features. The details of this case will be published elsewhere.[16]

Complete remission was achieved in seven of the eight AML patients, among whom patient no. 6 was still alive in the third remission at the time of the latest follow up (March 1987). Their survival times ranged from 5 to 31 months, with a median of 12.8 months. Patient no. 9 did not achieve

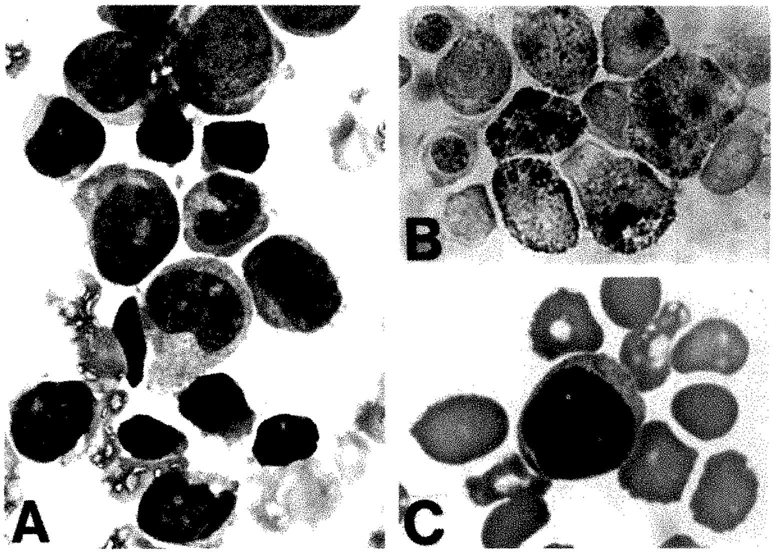

Fig 1. (A) Bone marrow aspirate from patient no. 4 showing myeloblasts and maturing granulocytic elements. (B) Myeloblasts from patient no. 4 showing peroxidase activity. (C) A myeloblast from patient no. 9 containing a single Auer rod in the cytoplasm.

remission and died 4 months after the onset of blastic crisis; her total survival time from initial diagnosis was 56 months.

Cytogenetic findings. The results of the chromosome analyses of the nine patients are summarized in Table 2. The t(7;11) translocation was detected in all of the patients. The breakpoints on chromosomes 7 and 11 were common to all patients and occurred at 7p15 and 11p15, respectively (Fig 2). At the time of diagnosis, five of the eight AML patients exhibited the t(7;11) as the sole chromosome abnormality. Patient nos. 7 and 8 showed others in addition to t(7;11), the former showing 6q − and the latter showing 7q −. Karyotypically normal cells were observed in four of the eight AML patients, including patient no. 1 who exhibited complex additional abnormalities during the later clinical course. Patient no. 9 showed t(7;11) together with trisomy 8 and an extra Ph^1 chromosome in blastic crisis in addition to the Ph^1 translocation, which was the only chromosomal abnormality observed in the chronic phase.

DISCUSSION

Since the first description of the t(7p −;11p +) translocation in the blastic crisis of a patient with Ph^1-positive CML,[7] five patients with this abnormality have been reported[8-12] (Table 3). A review of 14 cases including the present nine indicates that this abnormality frequently occurs in AML patients (11/14 cases), particularly those with subtype M2 (7/14) and occasionally in M4 and M5, rather than in CML patients with or without the Ph^1 translocation (3/14). These findings contradict the previous report that suggested an association of t(7;11) with Ph^1-negative CML.[11] The occurrence of t(7;11) may be analogous to that of the t(8;21) abnormality because the latter has been reported to occur predominantly in subtype M2 and occasionally in M1 and M4.[17,18]

Our patients commonly showed decreased NAP levels, peroxidase-positive leukemic blasts, and Auer rods in a fraction of leukemic blasts (Table 1). These features are usually observed in patients with t(8;21).[18-20] Although large myelocytes and metamyelocytes, giant mature granulocytes, and abnormal eosinophils with basophilic granules were found in some of our patients, marrow eosinophilia was not observed in our patients. The presence of these abnormal bone marrow cells and marrow eosinophilia is another morphological features of an M2 patient with t(8;21).[18-20] Auer rods, occurring in about 30% of AML patients,[21] are thought to be a distinctive feature of myeloid malignancies, although they are quite rare in myeloblastic crisis of CML. In this context, special attention should be paid to patient no. 9 who showed Auer rods in a small fraction of blasts and low NAP

Table 2. Cytogenetic Findings of Nine Patients With t(7;11)

Case No.	Date	Specimen	Total No. of Metaphases	No. of Cells (%)	Karyotype
1	May 1985	PB	5	4 (80.0)	46,XX,t(7;11)(p15;p15)
				1 (20.0)	46,XX
	September 1985	BM	30	12	46,X,t(X;13)(p21;p13),−21,del(6)(q23),t(7;11)(p15;p15),+der(21)t(21;?)(q22;?)
				9	46,XX,−21,del(7)(p12),del(11)(p14),del(15)(q15),t(2;9)(q13;q34),+der(21)t(21;?)(q22;?)
				1	46,XX,−21,del(7)(p12),del(11)(p14),+der(21)t(21;?)(q22;?)
				8	46,XX
	February 1985	BM	20	16	46,X,t(X;13)(p21;p13),−21,del(6)(q23),t(7;11)(p15;p15),+der(21)t(21;?)(q22;?)
				3	46,X,t(X;13)(p21;p13),−21,t(3;20)(q25;q12),t(7;11)(p15;p15),+der(21)t(21;?)(q22;?)
				1	46,XX
2	March 1985	BM	20	20 (100.0)	46,XY,t(7;11)(p15;p15)
3	March 1986	BM	18	8 (44.4)	46,XX,t(7;11)(p15;p15)
				10 (55.6)	46,XX
4	August 1985	BM	20	20 (100.0)	46,XX,t(7;11)(p15;p15)
5	March 1985	BM	20	20 (100.0)	46,XY,t(7;11)(p15;p15)
	April 1985	BM	7	7	46,XY,t(7;11)(p15;p15)
6	October 1984	BM	5	5 (100.0)	46,XY,t(7;11)(p15;p15)
7	September 1982	BM	93	65 (69.8)	46,XY,del(6)(p21),t(7;11)(p15;p15)
				28 (30.2)	46,XY
8	December 1985	BM	32	25 (78.1)	46,XY,del(7)(q32q35),t(7;11)(p15;p15)
				7 (21.9)	46,XY
9	July 1982	BM	10	10 (100.0)	46,XX,t(9;22)(q34;q11)
	November 1986	PB	60	37	47,XX,t(9;22)(q34;q11),t(7;11)(p15;p15),+Ph^1
	(M-BC)			14	47,XX,t(9;22)(q34;q11),t(7;11)(p15;p15),+8
				5	48,XX,t(9;22)(q34;q11),t(7;11)(p15;p15),+8,+Ph^1
				4	46,XX,t(9;22)(q34;q11),t(7;11)(p15;p15)

Abbreviations: M-BC, myeloid blastic crisis; PB, periperhal blood; BM, bone marrow.

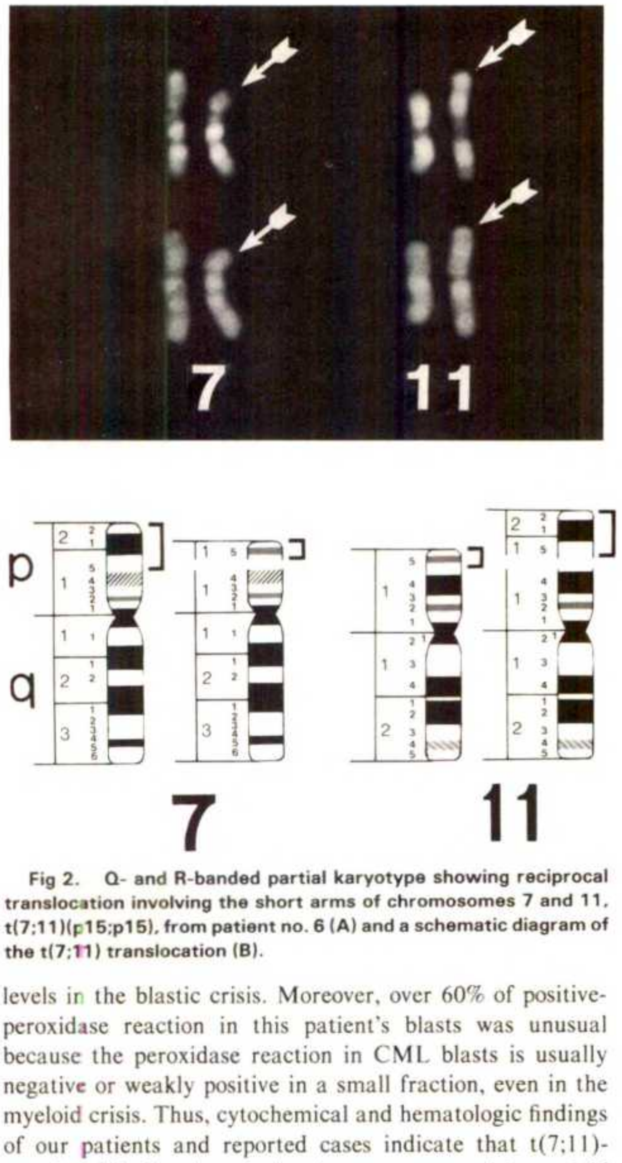

Fig 2. Q- and R-banded partial karyotype showing reciprocal translocation involving the short arms of chromosomes 7 and 11, t(7;11)(p15;p15), from patient no. 6 (A) and a schematic diagram of the t(7;11) translocation (B).

levels in the blastic crisis. Moreover, over 60% of positive-peroxidase reaction in this patient's blasts was unusual because the peroxidase reaction in CML blasts is usually negative or weakly positive in a small fraction, even in the myeloid crisis. Thus, cytochemical and hematologic findings of our patients and reported cases indicate that t(7;11)-associated leukemia may involve precursors with myeloid maturation, although other morphological features that distinguish this type of luekemia from others were not observed.

In the present study, AML patients exhibited a fairly good response to chemotherapy, although the CML patient failed to enter remission, as did two other CML patients reported in the literature (Table 3). However, the remission rate in our AML patients (7/8 cases, 87.5%) as well as the overall rate obtained when those reported in the literature were included (10/11 caess, 90.9%) is not significantly higher than that of M2 patients without t(7;11) at our institutes.

As shown in Tables 2 and 3, the t(7;11) translocation mostly occurred as the sole chromosome abnormality in the patients concerned herein. In the previously reported five patients with t(7;11), the breakpoint of chromosome 11, 11p15, concurred with that observed in our nine patients, whereas somewhat variable breakpoints (p13 to p15) were observed for chromsome 7. Recently, we have corrected the breakpoint of 7p14 to 7p15 in a previously reported CML case (patient no. 14) (Tomiyasu et al, unpublished). In addition, the 7p13 breakpoint in another reported case (patient no. 12), was revised to 7p15 (Ishihara et al, unpublished). Thus, 13 of the 14 patients thus far reported show the same 7p and 11p breakpoints in the t(7;11) translocation. A complex translocation involving chromsomes 7 and 11 at these breakpoints has recently been reported as the sole abnormality in chronic phase of a Ph^1-negative CML patient[22] in whom a low NAP level and peroxidase positivity were also observed. This patient may therefore bear a variant type of the standard t(7;11) translocation.

Because the band 11p15 includes the c-Ha-*ras*-1 oncogene locus,[23] it will be interesting to assess the involvement of this oncogene in the t(7;11) translocation. Our preliminary results from the in situ chromosome hybridization study showed a different localization for the c-Ha-*ras*-1 gene in the t(7;11) between one AML patient (patient no. 6) and one CML patient (patient no. 9) of the current series[24]; the translocation of c-Ha-*ras*-1 was demonstrated in patient no. 6 but not in patient no. 9. This may indicate a difference in the breakpoint within 11p15 at a molecular level between these two cases, as has been shown recently for the molecular breakpoints of 22q11 in Ph^1 translocation between CML and acute lymphocytic leukemia cases.[25] Further studies should be performed on more patients with t(7;11) to determine both its c-Ha-*ras*-1 localization and the involvement of this oncogene in the leukemic process.

Table 3. Clinical, Hematologic, and Cytogenetic Findings of the Patients With t(7;11) Reported in the Literature

Case No.	Age/Sex	Diagnosis	Auer Rods	NAP level	POX	AS-D ch	α-NB	Karyotype	RM	Survival*	Reference
10	25/F	AML + MF	+	276	+	–	–	46,XX,t(7;11)(p15;p15)	Yes	17 mo	10
11	72/F	AML (M4)	–	ND	+	–	–	46,XX,t(7;11)(p14;p15)	Yes	8 mo	9
12	18/F	AML (M4)	–	ND	+	+	–	46,XX,t(7;11)(p13;p15)	Yes	20 mo	12
13	6/F	Ph^1(–) CML-BC	?	8				46,XX,t(7;11)(p15;p15)	No	5 d	11
14	37/M	Ph^1(+) CML-CP	–	38				46,XY,t(9;22)(q34;q11)	No	34 m (4 m)	8
		pH^1(+) CML-BC	–	ND				46,XY,t(9;22)(q34;q11),t(7;11)(p14;p15)			

*The number in the parenthesis shows the survival time from blastic crisis.

†The 7p breakpoint in these cases recently has been revised to be 7p15 (see the text).

In conclusion, the present study indicates that a t(7;11)-associated leukemia represents a subtype of myeloid malignancy showing characteristic clinicohematologic features as described earlier. Considering that all of the 14 patients with t(7;11) thus far reported are Japanese, its occurrence might show an uneven geographic distribution, as has already been found for the t(8;21) and t(15;17) translocations, both of which showed a much higher incidence in Japan than in other countries.[6,7,26] Nevertheless, careful observation may help to detect leukemia patients with t(7;11) in other countries, particularly because two non-Japanese patients with myeloid malignancy have recently been reported to have the t(7;11) abnormality.[27]

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Philadelphia-Positive Chronic Myeloid Leukemia With a Chromosome 22 Breakpoint Outside the Breakpoint Cluster Region

By Licia Selleri, Franco Narni, Giovanni Emilia, Alessandro Colò, Patrizia Zucchini, Donatella Venturelli,
Amedea Donelli, Umberto Torelli, and Giuseppe Torelli

In chronic myelogenous leukemia (CML) the reciprocal translocation resulting in the Philadelphia chromosome (Ph^1) leads to the formation of a chimeric transcriptional unit carrying both c-*abl* and *bcr* genetic information whose transcript is a new fused mRNA of 8.5-kilobases (kb) and whose translational product is a 210-kD phosphoprotein with tyrosine kinase activity implicated in the pathogenesis of CML. Twenty patients affected by Ph^1-positive CML were studied by Southern blot analysis with *bcr*. Unexpectedly, in three Ph^1-positive patients, the breakpoint of chromsome 22 was located neither inside the *bcr* region nor 5′ to it. Northern blot analysis of the RNAs of two of these patients showed the absence of a detectable 8.5-kb chimeric mRNA. In the third patient a chimeric mRNA was detected by a c-*abl* cDNA probe but failed to hybridize with a *bcr* cDNA probe and showed very low hybridization levels with further 5′ *bcr* cDNA probes. The possibility is raised that in CML a breakpoint outside *bcr* might either still allow the formation of a chimeric mRNA, possibly through alternative splicing mechanisms, or might prevent the transcription of the chimera. In the latter case different molecular events resulting in the formation of a Ph^1 chromosome may underlie the same myeloid neoplastic phenotype.

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THE HALLMARK OF CHRONIC myeloid leukemia (CML) is the presence of a shortened chromosome 22, the Philadelphia (Ph^1) chromosome, in more than 90% of cases.[1] The Ph^1 chromosome, which can be considered the first karyotypic marker consistently present in a human neoplasia, is the result of a reciprocal translocation between chromosomes 9 and 22, t(9;22) (q34;q11).[2] This translocation moves the c-*abl* protooncogene from chromosome 9 to chromosome 22[3] and the c-*sis* protooncogene from 22 to 9.[4]

The breakpoint of chromosome 9 has been found to occur at variable sites in a region of up to 100 kilobases (kb) 5′ upstream from the first v-*abl*-related human c-*abl* sequence.[5] On the other hand, the breakpoint of chromosome 22 has been shown to be clustered within a limited region of about 5.8 kb, the breakpoint cluster region (*bcr*),[6] which is the middle portion of a transcribed gene of unknown function.[7]

The reciprocal translocation therefore brings the 5′ end of the c-*abl* oncogene into head-to-tail juxtaposition with the truncated 3′ portion of *bcr* on chromosome 22. This mechanism leads to the formation of a new chimeric transcriptional unit containing genetic information from both c-*abl* and *bcr* genes[7] whose resulting transcript is a new fused mRNA of about 8.5 kb.[8]

The translational product of this hybrid mRNA is a 210-kD phosphoprotein with tyrosine kinase activity. This protein is larger than the normal p145 kD c-*abl* protein because of a replacement of aminoterminal c-*abl* sequences by *bcr* sequences.[9] Interestingly, the aminoterminal alteration is in fact responsible for the transforming activity of v-*abl* in Abelson murine leukemia virus.[10] This seems to raise the question whether the altererd *abl* protein may play a role as a transforming protein in the pathogenesis of CML.

Therefore the most important genomic event in CML seems to be the juxtaposition of c-*abl* with the broken *bcr* gene. Although the breakpoint on chromosome 9 is quite variable and therefore the length of the hybrid *bcr*/*abl* gene also varies, the fused mRNA is consistent from patient to patient as a result of primary transcript splicing.[8]

It is now well established that in Ph^1-positive acute lymphocytic leukemia (ALL) the breakpoint of chromosome 22 can take place outside the *bcr* region.[11] In such cases a novel 6.5-kb chimeric mRNA[12] and a novel 190-kD chimeric protein[12-14] were observed. On the other hand, some observations seemed to suggest that perhaps in CML too the breakpoints on chromosome 22 are more heterogeneous than previously recognized.[6,15] This focused our attention on the importance of analyzing as many as possible Ph^1-positive CML patients to examine, by Southern blot analysis with a *bcr* probe, how large a variation there could be in the location of chromosome 22 breakpoints that still allows the formation of the novel chimeric mRNA.

PATIENTS AND METHODS

Patients. Twenty adult patients affected by typical Ph^1-positive CML were studied. In all cases leukapheresis procedures as well as bone marrow aspirations were performed after informed and explicit consent of the patient was obtained, as required by our institutional guidelines. Ten patients were in chronic phase, three were in accelerated phase, and 7 were in blast crisis (BC). Leukemic cells were obtained by leukapheresis.

Cytogenetic studies. Cytogenetic studies were performed on bone marrow and/or peripheral blood cells after a 24-hour incubation in tissue culture media without mitogen, and standard trypsin Giemsa chromosome preparations were made. The karyotype was expressed by using the recommendations of the International System for Human Cytogenetic Nomenclature.[16]

Southern and Northern blots. Leukocyte-enriched plasma was diluted in RPMI and centrifuged to obtain cell pellets from which nucleic acids were extracted. DNA was isolated according to the technique of Gross-Bellard et al with minor modifications[17] Control DNA was extracted from leukocytes of two normal blood donors.

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Ten micrograms of each DNA was digested with the restriction endonucleases *Bam*HI, *Bgl*II, *Bam*HI together with *Bgl*II, *Eco*RI and *Hin*dIII; run in 0.7% agarose gel electrophoresis; and then transferred to a Gene Screen (NEN) membrane with a standard Southern blot technique.[18]

RNA was extracted by following the technique described by Torelli et al.[19] Total RNA was then enriched for polyadenylate-containing RNA by one cycle of purification on oligo-dT-cellulose affinity chromatography.[20] Ten micrograms of poly (A)+ RNA were size fractionated by electrophoresis on 0.8% agarose gel containing 7% formaldehyde and then transferred to nitrocellulose filters as described.[20]

Preparation of DNA probes. To study the rearrangement of *bcr* we used a subgenomic probe represented by a *Hin*dIII-*Bgl*II genomic restriction fragment of 1.2-kb (Pr-1, Oncogene Sciences, Inc, Mineola, NY) that corresponded to an intron region of the gene located in the middle of the *bcr* region[6] and two contiguous *bcr* cDNA fragments of 0.6 kb (6.13-0.6) and 0.3 kb (6.13-0.3) representing a coding portion of the gene 5′ to the *bcr* region. The cDNA fragments used to study the expression of the chimeric mRNA were represented by a 0.5-kb human *bcr* cDNA restriction fragment (Pr-2, Oncogene Sciences) isolated from cDNA V1-3[21] and by *bcr* cDNA probes (6.13 and K31) corresponding together to a coding segment of about 1.4 kb 5′ to the sequences represented by the *bcr* probe Pr-2. Furthermore, a human c-*abl* cDNA fragment of 1.7 kb (K38) corresponding to the 3′ end of the c-*abl* cDNA was used.

The probes 6.13, K31, and K38 were developed and generously provided by Dr G. Grosveld, Erasmus University, Rotterdam, The Netherlands. A c-*sis* probe corresponding to a 1.7-kb *Bam*HI subgenomic fragment cloned into pBR 322[22] was used to examine whether a possible breakpoint had occurred in the subbands q12.3 to q13.1. A C_λ probe corresponding to a 0.8-kb *Eco*RI subgenomic fragment containing the $C_{\lambda 1}$ light-chain gene[23] was used to study the presence of a possible breakpoint in the subband q11.12.

To define as precisely as possible that some of the cases pertained to the myeloid lineage, a probe for C_μ was used that corresponded to a 1.2 *Eco*RI restriction fragment containing a part of the first and all of the second and the third exon of the Ig heavy-chain μ gene.[24] A probe for T cell receptor (TCR) β was further used and corresponded to a 0.77-kb insert containing J and C regions cloned in the *Pst*I site of pBR 322.[25]

All the probes were nick-translated with α^{32}P deoxycytidine triphosphate (dCTP) to a specific activity of about 2×10^8 cpm/μg DNA.

Hybridization and autoradiography. Southern and Northern blot hybridizations were performed according to procedures already described in detail.[26] Washing of the Southern blot filters was carried out in 0.3× SSC at 65°C. Washing of the Northern blot filters was performed in 0.1× SSC at 65°C. Autoradiography was performed at −80°C with intensifier screens for two to seven days.

RESULTS

All 20 adult patients studied were affected by CML diagnosed according to French-American-British (FAB) morphological and cytochemical criteria.[27] Ten patients were in fact in chronic phase, three in accelerated phase, and seven in BC. Cytogenetic analysis of all patients showed the typical t(9;22) translocations, except in one case in which a simple variant translocation of the type t(22;22) was present. Most patients in BC showed numerical chromosomal abnormalities (hyperdiploidy) and/or Ph[1] duplication as expected.[28]

No c-*abl* rearrangement was detected in any of the cases studied,[29] which did not seem surprising because the chromosome 9 breakpoints can occur in a region very far upstream to the first c-*abl* exons. Concerning chromosome 22 breakpoints, restriction data of 17 of 20 patients together with cytogenetic data have already been discussed in detail elsewhere[29] and are consistent with the results already reported in the literature.[6,7]

In three cases rather unexpectedly (T.V., R.G., and Q.S., all in chronic phase), no rearranged bands were detectable after digestion of the DNA with the restriction endonucleases *Bam*HI, *Bgl*II, *Hin*dIII, *Eco*RI (Fig 1), and *Bam*HI together with *Bgl*II (not shown). In fact, with all the aforementioned restriction enzymes in all three cases, the hybridization with the 1.2-kb *Hin*dIII-*Bgl*II *bcr* genomic fragment showed only the normal germ line band as in the control DNA from normal leukocytes; they measured, respectively, 3.3 kb in the *Bam*HI-digested DNAs, 5.0 Kb in the *Bgl*II-digested DNAs, 4.5 kb in the *Hin*dIII-digested DNAs, 23 kb in the *Eco*RI-digested DNAs (Fig 1), and 2.1 kb in the same DNAs digested with *Bgl*II together with *Bam*HI. These experiments were performed twice for all three patients with different blood samples collected within an interval of few months. In all three cases both Ig heavy-chain and TCR β chain genes were in a germ line configuration (data not shown), which confirmed, in addition to the

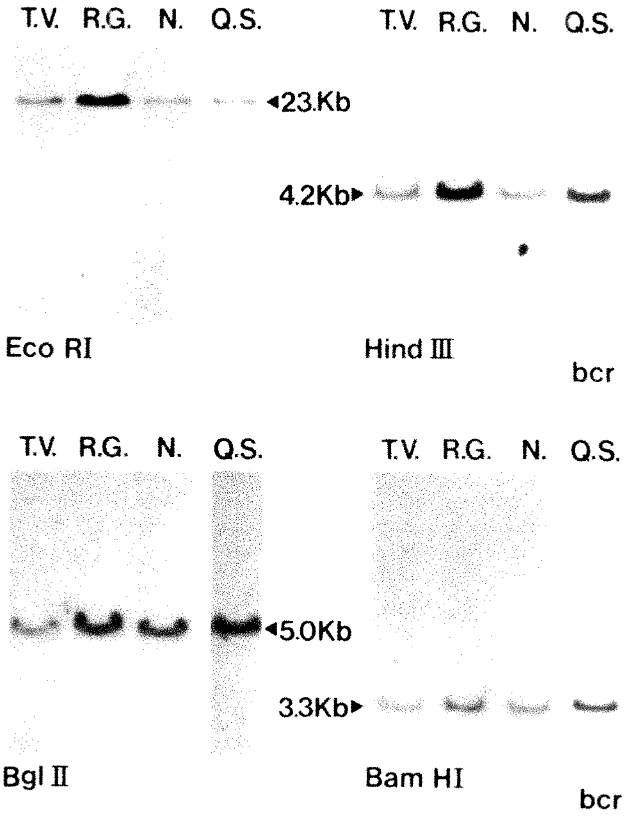

Fig 1. Southern blot analysis of the DNAs of the three patients (T.V., R.G., and Q.S.) that were digested with *Eco*RI, *Hin*dIII, *Bgl*II, and *Bam*HI and hybridized to the 1.2-kb *Hin*dIII-*Bgl*II *bcr* genomic fragment (Pr-1). No rearranged bands are present in any of the cases with all the restriction enzymes. Only the normal band, as in control DNA of normal leukocytes (N), is present with all the enzymes in all three cases.

FAB morphological and cytochemical data, that patients' leukemic cells were of the myeloid lineage.

Our findings suggested that in these three patients the breakpoint was located either 5′ or 3′ to the *bcr*, but certainly not within it despite the fact that in all three cases a t(9;22) translocation was present (Fig 2) in two successive cytogenetic analyses carried out within an interval of several months and despite the typical CML phenotype. It must be added that the peripheral cell populations studied were formed by a vast majority (>80%) of largely immature myeloid elements and that the Ph^1 translocation was observed in more than 90% of the metaphases analyzed in each case.

Because it was suggested that in Ph^1-positive ALL cases the breakpoints of chromosome 22 could be located 5′ to the *bcr* region,[11,30] we explored the possibility that a similar genomic event could have taken place in our CML patients. Therefore in Southern blot experiments we used the probes 6.13-0.6 (0.6 kb) and 6.13-0.3 (0.3 kb), which represent together 0.9 kb corresponding to the middle portion of the *bcr* cDNA sequences 5′ upstream the *bcr* region[21] (G. Grosveld, personal communication). Again the pattern obtained after digestion with *Bam*HI, *Bgl*II, and *Eco*RI was in all three cases identical to that of normal leukocyte DNA, both with the 0.6 kb probe (data not shown) and with the more 5′, 0.3-kb probe (Fig 3).

The DNAs of the three patients were then hybridized to the c-*sis* probe after digestion with *Eco*RI and *Hin*dIII and to the C_λ probe after digestion with *Eco*RI, *Bam*HI, and *Hin*dIII. No rearranged bands were found in any case, either with c-*sis* or with C_λ with all the restriction enzymes used (data not shown). With the c-*sis* probe, however, two of the three patients (T.V. and R.G.) exhibited a polymorphic pattern already described[31] after digestion with *Hin*dIII that gave bands of about 21.7 kb and 14.5 kb (not shown). Furthermore, in one of the three patients (R.G.) a polymorphic restriction pattern, already described,[31] was also observed in the *Eco*RI-digested DNA hybridized to the C_λ probe that gave an additional band of about 22 kb (not shown).

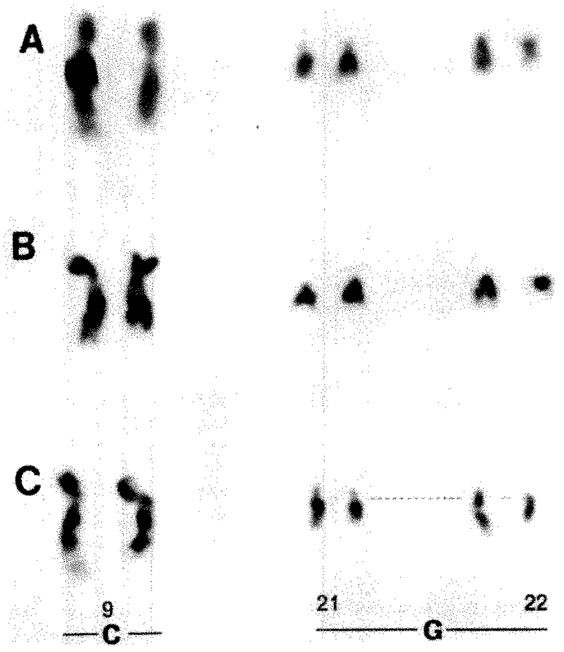

Fig 2. Partial karyotypes of the three patients showing a t(9;22) translocation in all cases. 1, the patient T.V; 2, R.G; and 3, Q.S.

As far as the expression of *bcr* is concerned, the poly (A)+ RNAs of the three described patients together with the poly (A)+ RNAs derived from two other classic Ph^1-positive CML cases were hybridized on the same filters with the *bcr* cDNA probe (Pr-2) representing exons 1 and 2 of the *bcr* region and at least two further *bcr* exons 5′ upstream to them. In all cases two faint bands representing normal *bcr* mRNA species were detected (Fig 4). A major 8.5-kb mRNA species was detectable only in the RNAs analyzed as controls and was apparently absent in the RNAs of the three patients already mentioned (Fig 4) despite the fact that the chimeric mRNA, when present, is usually more abundant than the normal *bcr* mRNA transcripts.[8]

We then submitted the same RNAs to a further Northern blot analysis using a c-*abl* c-DNA probe (K38). This experiment revealed, both in poly (A)+ and total RNA of one case (T.V.), the presence, in addition to the normal expected bands, of a faint but still detectable band of about 8.5 kb while confirming the absence of a chimeric mRNA species in the remaining two cases (Fig 5). The results obtained in patient T.V. led us to investigate whether the chimeric mRNA might be detected by *bcr* cDNA probes representing coding sequences located more 5′ to those represented by Pr-2. In fact Northern blot analysis of the poly (A)+ RNA of patient T.V., which was carried out with the *bcr* cDNA probes 6.13 and K31 and represented together about 1.4-kb sequences 5′ to the Pr-2 sequences, revealed extremely faint bands, one of which apparently corresponds to the chimeric mRNA present in a classic CML case (Fig 6). The same experiment did not show any detectable level of chimeric mRNA in patient Q.S. (Fig 6) or patient R.G. (not shown).

The densitometric analysis did not reveal relevant differences in the amount of RNA present in each lane in all experiments.

DISCUSSION

The analysis of our data led us to the conclusion that in these three patients affected by classic Ph^1-positive CML the breakpoint of chromosome 22 was certainly outside the *bcr* region. This finding seemed rather unusual, even though in the literature some evidence has already been reported of Ph^1-positive CML in which a breakpoint inside *bcr* was not found,[15] and it has been suggested that in CML the chromosome 22 breakpoints must be more heterogeneous than previously recognized.[6,15] Southern blot hybridizations with the *bcr* (Pr-1) probe suggested that in these three cases the breakpoint should be located either 5′ or 3′ to the *bcr* region.

Meanwhile, the absence of rearranged bands in the DNAs digested with different enzymes and hybridized with the probes Pr-1, 6.13-0.6, and 6.13-0.3 prevented us from localizing the breakpoint in the coding segments 5′ to the *bcr* in a region of about 13 kb flanking the 5′ site of the *bcr*.

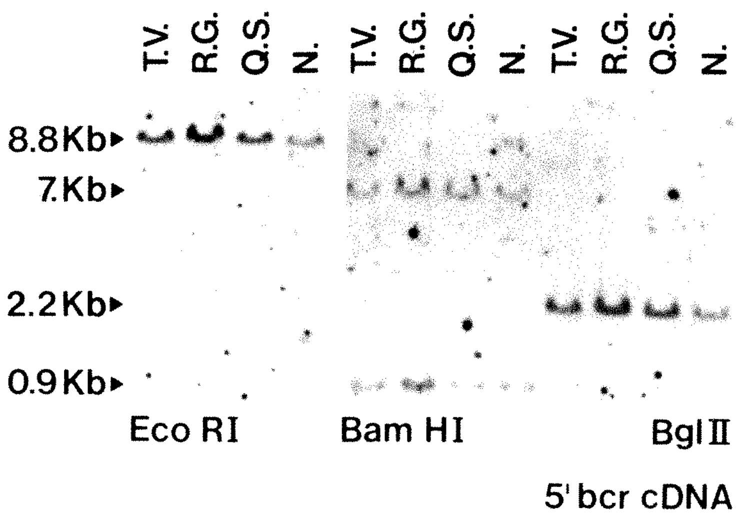

Fig 3. Southern blot analysis of the DNAs of the three patients (T.V., R.G., and Q.S.) that were digested with *Eco*RI, *Bam*HI, and *Bgl*II and hybridized to the 0.3-kb 5' *bcr* cDNA (6.13-0.3) probe. The restriction pattern with all the enzymes in all three cases is identical to that of normal leukocyte DNA (N). A faint hybridization signal of a previous hybridization of the same filter with probe 6.13-0.6 is still detectable in the region of about 8.0 kb in the DNAs digested with *Bgl*II.

Consequently, we reasoned that in these three cases the breakpoint should be located either in the far 3' extremity of the *bcr* gene or outside the gene.

The absence of rearranged bands with both C$_\lambda$ and c-*sis* probes made it clear that the breakpoint had occurred neither in the C$_\lambda$ locus located in the q11.12 subband nor in the c-*sis* locus harbored in the q12.3-13.1 subbands. At present we cannot explain the apparently high frequency of polymorphism of both these loci in these three cases.

With respect to the Northern blot hybridization data, we did not find a chimeric mRNA of 8.5 kb in two of these three Ph^1-positive CML patients. We are well aware of the uncertainties related to a quantitative evaluation of mRNA expressed at low level; nevertheless, our results indicate that in these two cases the level of transcription of the 8.5-kb mRNA, if present, must be at least much lower than in common Ph^1-positive CML. These findings seemed rather unusual because data reported in literature show the presence of this chimeric mRNA in practically all cases of Ph^1-positive CML studied thus far with rare exceptions.[32,33]

So the possibility is raised that a breakpoint of chromosome 22 outside the *bcr* region might result in a novel genomic structure that does not allow the transcription of a 8.5-kb chimeric mRNA, at least at a detectable level, while leaving unaffected the formation of the mRNA species presumed to be normally transcribed by the *bcr* and *abl* genes.[21]

A similar genomic event has been described in several patients affected by ALL with a t(9;22) translocation in which the *bcr* region was not involved in the chromosomal rearrangement.[11-14,30] By analogy with the reports in some Ph^1-positive ALL cases,[12-14] it would be worthwhile in these two cases to look for the presence of *abl*-derived kinases different from the chimeric p210 and from the normal p145 while taking in mind that the cells of these two cases are certainly not lymphoid.

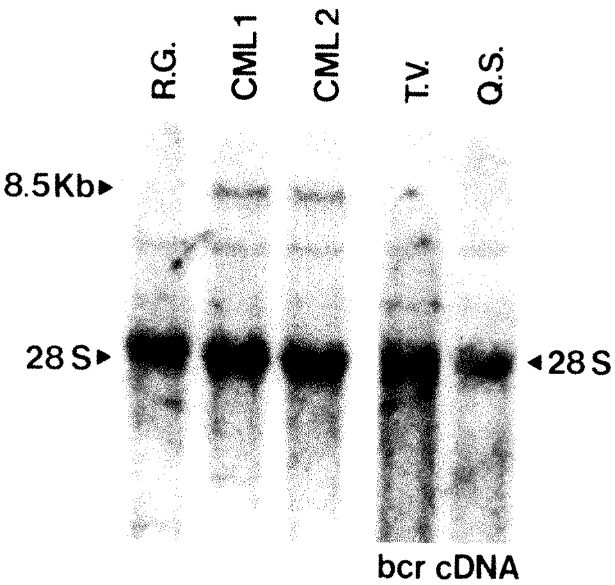

Fig 4. Northern blot analysis of the poly (A)+ RNAs of the three patients (R.G., T.V., and Q.S.) hybridized to the *bcr* cDNA probe (Pr-2). The 8.5-kb chimeric mRNA is clearly detectable only in the two CML populations used as controls (CML 1 and CML 2). The normal *bcr* mRNA species are present in all the populations.

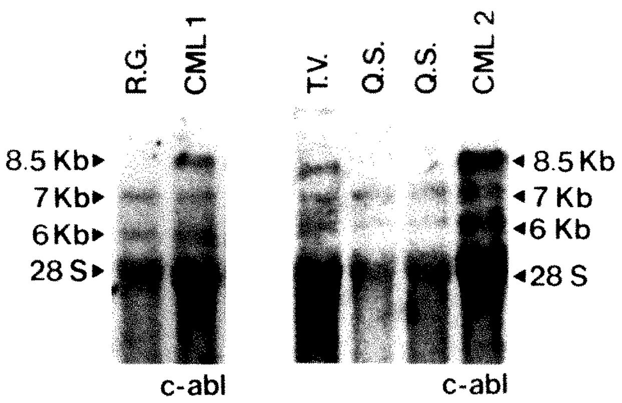

Fig 5. Northern blot analysis of the poly (A)+ RNAs of the three patients (R.G., T.V., and Q.S.) hybridized to the c-*abl* cDNA probe (K38). The 8.5-kb chimeric mRNA is clearly detectable only in the two CML populations used as controls (CML 1 and CML 2) and in one of the three patients (T.V.). The normal *abl* mRNA species of 6 and 7 kb are present in all the RNA populations.

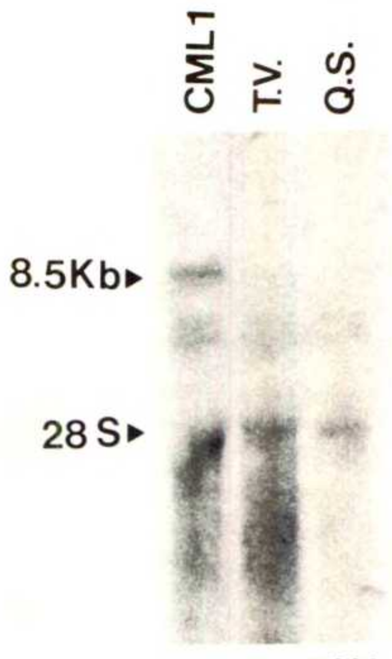

Fig 6. Northern blot analysis of the poly(A) + RNAs of patients T.V. and Q.S. that are hybridized to the 5′ *bcr* cDNA probes. The 8.5-kb chimeric mRNA is clearly detectable in the CML population used as control (CML 1). In patient T.V. an extremely faint band, apparently corresponding to the chimeric mRNA of CML 1, is detectable. In patient Q.S. no hybridization of chimeric mRNA is detectable. Normal *bcr* mRNA species are present in all the populations.

The third case (T.V.), in which the c-*abl* probe reveals the chimeric 8.5-kb mRNA, seems to suggest that unusual breakpoints sometimes may still allow the formation of a chimeric mRNA. The same mRNA species shows an extremely low level of hybridization with 5′ *bcr* cDNA sequences and apparently no hybridization at all with the 0.5-kb *bcr* probe representing only the sequences immediately flanking the breakpoint. These findings seem to suggest that the *bcr* portion of the chimera is in some way different from the classic one, possibly as a result of molecular events like deletions or alternative splicing.[34]

Because different molecular patterns underlie the same clinical features, it can be only suggested that the relationship between the genetic alteration of the Ph[1] event and the resulting neoplastic phenotype remains to be elucidated. While this manuscript was under preparation, other authors have reported identical molecular abnormalities., ie, *bcr* rearrangement as in Ph[1]-positive CML, in Ph[1]-negative CML cases with and without the classic clinical features of CML. They suggest that factors other than the *bcr-abl* rearrangement might underlie the heterogeneous clinical features of CML[35]

In view of all these results it will be necessary to carefullly define the genomic structure, the mRNA transcripts, and the presence of the related proteins in a larger number of CML patients.

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The authors want to thank Dr Gerard Grosveld, Erasmus University, Rotterdam, for helpful suggestions, for the gift of the 6.13, K31, and k38 probes, and for kind revision of the manuscript; we would also like to acknowledge the expert technical assistance of B.M. Sala.

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Establishment and Characterization of a Human Myeloid Cell Line From Philadelphia Chromosome–Negative Myeloblastic Leukemia Arising in a Patient With Myelodysplastic Syndrome

By Todd M. McCarty, S. Rajaraman, F.F.B. Elder, P. Gadson, and E. Brad Thompson

A new hematopoietic cell line derived from a patient with Philadelphia chromosome (Ph^1)-negative myeloblastic leukemia arising from a form of myelodysplastic syndrome (MDS) is described. This cell line, designated TMM, consists of immature cells with the morphological characteristics of young myeloblasts and grows in suspension culture with a doubling time of about 30 hours. By cytochemical analysis the cultured cells were positive for acid phosphatase. They were free of the Epstein-Barr virus–associated nuclear antigen as well as terminal deoxynucleotidyl transferase. Further phenotypic analysis revealed the expression of the myelomonocytic-specific antigen Leu-M1 and receptors for the Fc portion of IgG. Partial differentiation of these cells could be induced by dimethyl sulfoxide, tetradecanoyl phorbol acetate, or hypoxanthine and resulted in cells of the myeloid series expressing lysozyme and receptors for the C3b complement protein. The karyotype was 46,XY, lacked the Ph^1 chromosome, and displayed no abnormalities at the light microscopic level. No rearrangement of the *bcr-c-abl* gene complex was found. This cell line should be useful for studying an important type of the heterogeneous population constituting Ph^1-negative myeloblastic leukemia, arising in this instance from MDS, as well as for studying differentiation and proliferation of human pluripotent stem cells.

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THE USE OF MODEL SYSTEMS to study human malignancies in vitro has been a productive approach in understanding the development, progression, and response to treatment of these disorders. But with the diversity of malignant disease collectively comprising the leukemias, no single model system has been satisfactory for all and in fact might be misleading for many. It is therefore important to examine various in vitro models representing the various specific categories of leukemia so as to better understand the mechanisms involved.

Dysplasias involving cells of mono/myeloid origin typify this diversity because they consist of distinct as well as ill-defined categories of disease. Although the Philadelphia chromosome (Ph^1) is found in about 90% of chronic myeloid leukemia (CML)[1] the fundamental nature of this disorder is unresolved. The remaining 10%, traditionally designated Ph^1-negative CML, is recognized as a biologically distinct subgroup.[2] More generally termed "a typical myeloproliferative disorder," this subgroup consists of a poorly understood heterogeneous population corresponding to much poorer prognoses.[2-4] In addition, a syndrome of myelodysplasia (MDS) having several subcategories has been defined in recent years, and patients with this syndrome have a high probability of developing leukemia.[5-18] Unfortunately, our ability to study this area of disease is limited, and a permanent in vitro model would be a valuable tool in understanding the nature of this disorder.

Although several Ph^1-positive CML cell lines have been established,[19-21] attempts to establish a Ph^1-negative CML cell line have been unsuccessful.[22] We are unaware of any cell lines having been established from patients with myelodysplastic syndromes (MDS).[23,24] Many myeloid cell lines originally expressing the Ph^1 chromosome have subsequently lost this marker, but lymphoblastoid cells carrying the Epstein-Barr virus resulted.[25-27] Here we describe the establishment and characterization of a Ph^1-negative CML cell line derived from a patient originally diagnosed as having Ph^1-negative CML in blast crisis. In review, the case history of the patient is consistent with an MDS progressing to a blastic phase. This cell line should prove beneficial in delineating the heterogeneity found within this category of disease, as well as in providing a new model system for studying differentiation and proliferation of human pluripotent stem cells.

MATERIALS AND METHODS

Case history. The TMM cell line was derived from the peripheral blood of a 62-year-old white male originally diagnosed as having Ph^1-negative CML in blast crisis. He was diagnosed as having chronic obstructive pulmonary disease in 1981 and treated with cephradine, vitamin K, theophylline, and terbutaline. In August 1984 he presented with a chronic buccal mucosa ulcer and was found to have thrombocytopenia (75,000/μL) and splenomegaly. His WBC was 17,100/μL with monocytosis (13%), and abnormal platelet morphology was observed. From that time until his death, the patient's blood cellularity was characterized by monocytosis (up to 59% on differential counts), frequently by eosinophilia (up to 24%), and by thrombocytopenia. In November 1984 he was diagnosed as having Ph^1-negative CML with a bone marrow karyotype of 46,XY, and a bone marrow biopsy that showed an M:E (myeloid:erythroid) of about 20:1, 5% to 10% myeloid blasts, numerous eosinophilic precursors, plus decreased and atypical megakaryocytes. Clinical discussions noted that this seemed an atypical case of CML, and in October 1985 the patient developed a marked increase in circulating blasts with progression of splenomegaly, pulmonary infiltrate, and anemia. He was treated with hydroxyurea and Depo-Testosterone. His WBC was 326,000/μL with 76% blasts, 1% banded neutrophils,

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1% segmented neutrophils, 17% monocytes, and 5% lymphocytes. Leukapheresis was performed for three consecutive days, and although the WBC dropped to 100,000/μL the patient continued to deteriorate and died on October 23, 1985, of bronchopneumonia.

Cell culture. The leukapheresed blood was collected in a heparanized bag on October 17, 1985, when the patient's WBC was 223,000 with 69% blasts. Mononucleated cells were separated by Ficoll/sodium diatrizoate (Sigma Chemical Co, St Louis) density gradient centrifugation and suspended in RPMI 1640 medium (GIBCO, Chagrin Falls, OH) supplemented with 10% heat-inactivated fetal calf serum (KC Biological, Lenexa, KS). After a lag phase, cells with blast morphology began to grow. There was no massive loss of cells, and the cultured line seemed to develop from many cells rather than a few clones. Cells were grown as stationary-suspension cultures at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells were maintained in logrithmic growth by manual dilution to densities between 10^5 and 5×10^5 cells/mL as determined by a model Zf Coulter counter (Coulter Electronics, Hialeah, FL) and by manual counting using a hemocytometer. Cell viability was determined by the exclusion of trypan blue (0.5%) after a five-minute treatment.

Morphological and cytochemical analysis. Cytocentrifuge preparations were stained with Wright-Giemsa in the University of Texas Medical Branch (UTMB) Special Hematology Laboratory for morphological analysis. The slides were examined by several members of the Department of Hematology and the experienced technicians of the Special Hematology Laboratory. For cytochemical analysis the following staining reactions were performed: peroxidase, α-naphthyl butyrate esterase (α-NBE), naphthol ASD chloroacetate esterase (CAE), acid phosphatase, alkaline phosphatase, PAS, and α-naphthyl acetate esterase (Sigma), with L cells used as a positive control.

Epstein-Barr virus–associated nuclear antigen (EBNA) terminal deoxynucleotidyl transferase. Testing for the presence of Epstein-Barr virus–associated nuclear antigen (EBNA) was kindly performed by Dr Keith Ramsey of the UTMB, Department of Internal Medicine, by the method of Reedman and Klein.[28] Raji cells were used as a positive control while K-562 cells were used as a negative control. Terminal deoxynucleotidyl transferase (TdT) was assayed by indirect immunoflourescence using the BRL TdT kit. (Bethesda Research Laboratories, Bethesda, MD)

Ultrastructural analysis. Unstimulated TMM cells and TMM cells stimulated to differentiate with hypoxanthine were fixed for electron microscopy in 1.25% glutaraldehyde in Dulbecco's phosphate-buffered saline (PBS, Sigma) for 30 minutes at room temperature. Localization of endogenous peroxidase was carried out according to the procedure of Graham and Karnovsky.[29] The cells were viewed by using a Philips EM 300 transmission electron microscope (Mahwah, NJ). Electron microscopy was carried out in the UTMB Cancer Center Core Facility.

Analysis of cell marker antigens. Cytocentrifuge preparations were air-dried for one hour, fixed in cold acetone for ten minutes, and stained after reaction with various antibodies by using the avidin-biotin-peroxidase complex method. The slides were incubated with monoclonal antibodies Leu-M1, Leu-14, Leu-11B, Leu-15, Leu-9, and anti-CR1 (Becton Dickinson, Sunnyvale, CA) and monospecific polyclonal antibodies to lysozyme and α1-antichymotrypsin (Dako Laboratories, Santa Barbara, CA) for 60 minutes at room temperature in a humidified chamber. Sheep antimouse IgG $F(ab')_2$, goat antimouse IgM $F(ab')_2$ or goat antirabbit IgG $F\ (ab')_2$ (Cooper Biomedical, Westchester, PA) and streptavidin-biotin-peroxidase complex (Amersham Corp, Arlington Heights, IL) were used as the second and third reagents. The final reaction product was visualized after incubation with diaminobenzidine, 0.05% in PBS, pH 7.2, containing 0.02% H_2O_2. A single-step procedure using peroxidase-conjugated rabbit antihuman Ig (polyvalent) was carried out to detect surface Ig. The reactions were scored based on the intensity of the color reaction: (−) no reaction, (+) clearly positive, (++) strongly positive. When a positive reaction was seen, virtually all cells on the slide were positive. Controls included (*a*) omission of the primary antibody, (*b*) substitution of the primary antibody with preimmune serum of the same species, and (*c*) omission of primary and secondary antibodies.

Differentiation induction. Cells were suspended in growth medium containing various inducing agents and incubated for six days under our standard culture conditions. Dimethyl sulfoxide (DMSO) and tetradecanoyl phorbol acetate (TPA) were obtained from Sigma, St. Louis and hypoxanthine from Calbiochem-Behring Corp. (La Jolla, CA). DMSO was diluted to a concentration of 180 mmol/L in RPMI 1640, and TPA was dissolved in absolute ethanol and diluted in RPMI 1640 to a concentration of 10^{-7} mol/L. Hypoxanthine was dissolved in boiling serum-free RPMI 1640 medium at a concentration of 50 mol/L and immediately diluted for use to 5 mmol/L. The cells were suspended at a density of 10^5 cells/mL in each inducing agent and monitored throughout the six-day period. At the end of that time the cells were stained with Wright-Giemsa for morphological analysis. Cell morphology was evaluated by the experienced staff of the UTMB Special Hematology Laboratory who were unaware of the various treatments the cells had received as the slides were read. Functional analysis was determined by the expression of cell marker antigens.

Cytogenetic analysis. TMM cells were treated with Colcemid (Boehringer-Mannheim, Indianapolis) at a final concentration of 5×10^{-3} μg/mL for 40 minutes. The cells were then subjected to hypotonic treatment (0.075 mol/L KCl) for 15 minutes at 37°C, fixed, and air-dried to slides by following conventional cytogenetic methodology. Trypsin G-banding followed methods modified from Seabright.[30]

Restriction endonuclease analysis. DNA was extracted from cells, digested with endonuclease, subjected to electrophoresis in agar gels, and transferred from the gels to nitrocellulose filter paper as previously described.[31] The filters were probed with a 0.65-kilobase (kb) clone of *c-abl* or a 1.2-kb clone of *bcr* (both obtained from Oncogene Sciences, Inc, Mineola, NY) and labeled to a specific activity of $>10^8$ cpm/mg by a standard nick-translation technique.[32] Hybridization conditions were as described previously.[31]

RESULTS

Establishment of TMM cell line. The patient's leukapheresed cells were placed in culture medium at a density of 5×10^5 cells/mL and left undisturbed for 1 week. During that time about 30% of the cell population became pyknotic and nonviable. Cells that did not adhere to the growth chamber were removed and placed into fresh culture medium. Small aggregates of viable cells were evident at that time, and the first subculture was made. In the following 2 weeks about 60% of the cell population was viable, and the cell aggregates were actively proliferating. During the next 13 weeks the cells were manually diluted once a week to a density of 10^5 cells/mL and fed with fresh medium. After 16 weeks in culture medium the cells were 95% viable and had a doubling time of about 30 hours. Since that time they have been stable. The cells that have been in continuous culture for over 8 months continue to have a doubling time of about 30 hours and grow as a mix of single cells and free-floating aggregates.

Morphological analysis. The patient's cells originally received in our laboratory were of myeloblastic appearance, and the differential count of peripheral blood included some mature neutrophils and monocytes. The blasts had round or lobulated nuclei with basophilic cytoplasm and a few vacuoles (Fig 1A). The morphological appearance of TMM cells was consistently that of immature, undifferentiated blasts resembling the original leukemic cells. The cytoplasm was basophilic, devoid of granules, and contained a few vacuoles. The nuclei were spherical and contained fine chromatin strands that stained reddish purple. The nuclear-to-cytoplasmic ratio was high, and one to five nucleoli with sharply defined borders were discernible (Fig 1B).

Cytochemical analysis. Cytochemical analysis of the original leukapheresed leukemic cells resulted in 58% of the cells staining positive for peroxidase, 79% for α-NBE, and 100% for acid phosphatase. TMM cells were 100% positive for acid phosphatase, with a punctate pattern observed, but were negative for α-NBE. Both the original leukemic cells and TMM cells were negative for TdT. TMM cells were negative for EBNA (Table 1).

Ultrastructural analysis. Electron microscopy studies revealed that TMM cells contain the large, spherical nuclei typical of blasts. In the cytoplasm a few vacuoles together with other usual cytoplasmic structures such as mitchondria, rough endoplasmic reticulum, and microtubule organizing centers were seen (Fig 2A). No viral structures were found. The TMM cells treated by hypoxanthine (5 mmol/L) were smaller and had indented to segmented nuclei and more mature cytoplasm. Many spherical, homogenously electron-dense, peroxidase-positive cytoplasmic granules were present, similar in appearance to those of normal granulocytic cells (Fig 2B).

Table 1. Cytochemical Analysis of Original Leukapheresed Leukemic Cells and TMM Cells

	Original Leukapheresed Leukemic Cells (%)	TMM Cells (%)
CAE	0	0
α-NBE	79	0
Peroxidase	58	3
Acid phosphatase	100	100
Alkaline phosphatase	0	0
PAS	0	0
EBNA	NT	0
TdT	0	0

Abbreviation: NT, not tested.

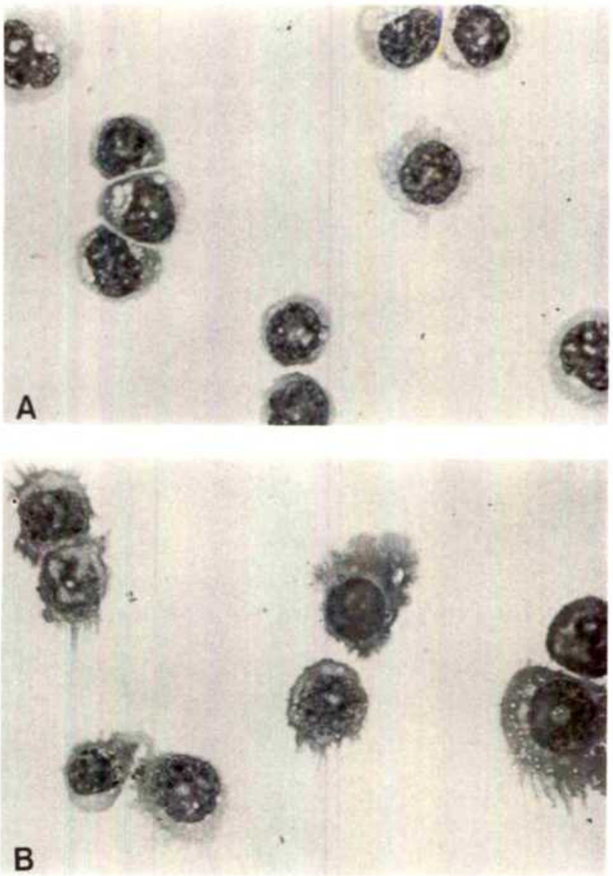

Fig 1. (A) Cytospin preparation of TMM cells stained with Wright-Giemsa (original magnification × 225; current magnification ×110). (B) Cytospin preparation of original leukapheresed leukemic cells stained with Wright-Gimesa (original magnification × 225; current magnification × 110).

Cell marker analysis. Both the original cells and TMM cells were surface Ig–negative, B-cell associated antigen (Leu-14)–negative, E rosette (Leu-9)–negative, Fc receptor (Leu-11b)–positive, myelomonocytic antigen (Leu-M1)–positive, α_1-antichymotrypsin–negative, C3b receptor (anti-CR1)–negative, and C3bi receptor (Leu-15)–negative. The original cells were positive for lysozyme, whereas TMM cells were negative (Table 2).

Differentiation induction. The uninduced TMM cells contained 2% mature myeloid cells. TPA and hypoxanthine each stimulated partial morphological maturation (42% and 68%, respectively) to cells with the appearance of early myeloid precursors while also inducing lysozyme and receptors for the C3b complement protein. TPA treatment did not stimulate increased adherence to the surface of the culture vessel but did slightly increase the tendency for cells to adhere to another. TPA did not induce α-naphthyl acetate esterase. Neither hypoxanthine nor TPA caused any of the cells to take on the appearance of monocyte/macrophage-like cells. DMSO also stimulated the cells to express lysozyme, but did not induce morphological maturation (Table 2).

Cytogenetic analysis. Cytogenetic analysis of 25 G-banded metaphase cells showed a range of 43 to 46 chromosomes. The modal number was 46 (22 of 25 cells), and the karyotype was 46,XY (Fig 3). The remaining three cells had karyotypes of 45,XY,−12, 44,XY,−7,−12; and 43,XY,−12,−21,−22, respectively. Scoring of 100 randomly selected metaphases showed 2% of the cells to be in the tetraploid range.

Lack of bcr-c-abl *complex rearrangement.* Although TMM cells lack the Ph^1 chromosome, instances are known in which euploid cells show rearrangement of the c-*abl* gene and *bcr* gene. Restriction endonuclease digestion and Southern blot analysis of DNA from TMM cells and the leukemic

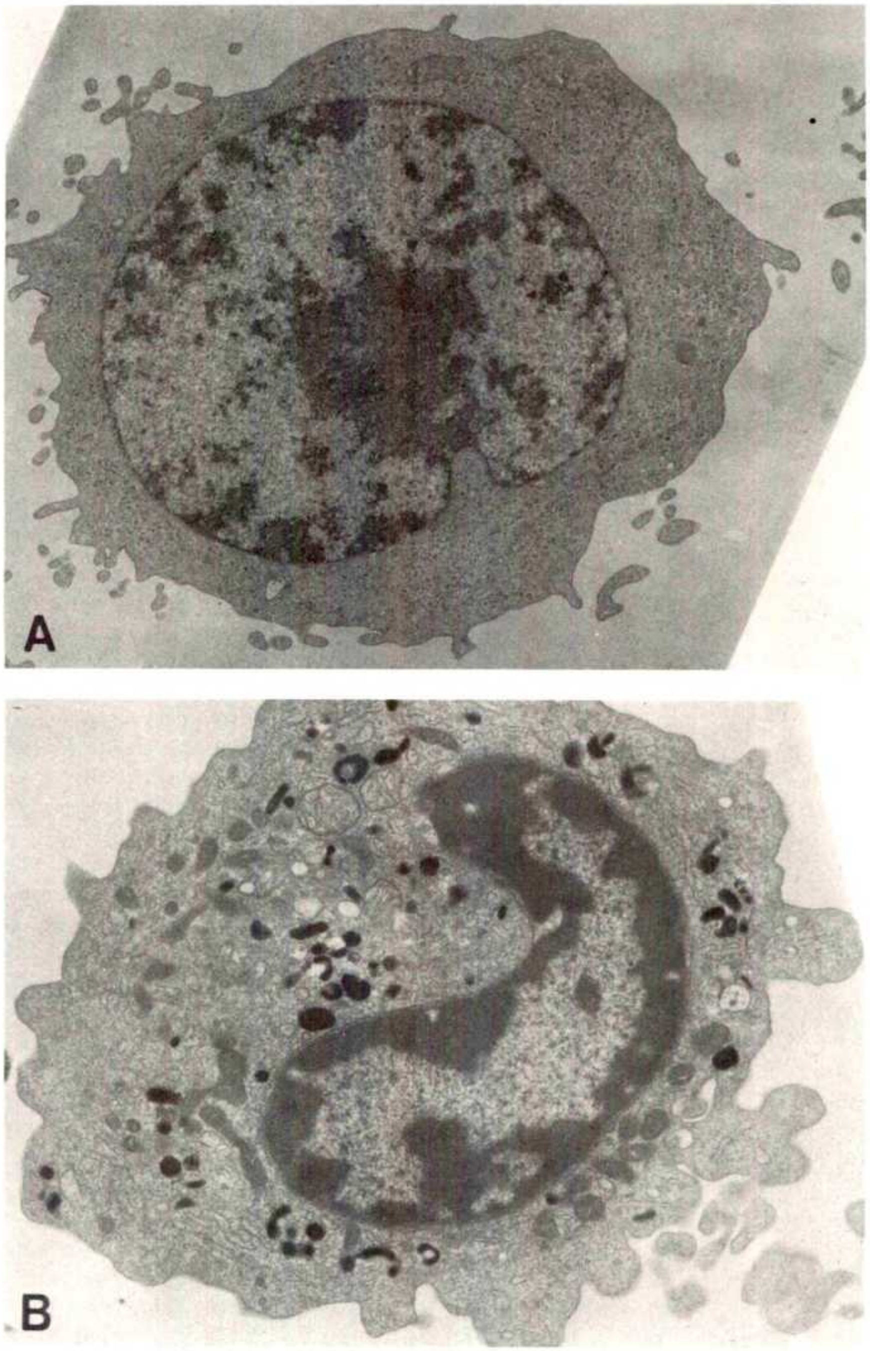

Fig 2. (A) Ultrastructural appearance of a typical TMM cell cultured under usual conditions (original magnification × 12,000; current magnification × 9,000). (B) Ultrastructural appearance of a typical TMM cell cultured in hypoxanthine (5 mmol/L) for six days (original magnification × 20,000; current magnification × 15,000).

Table 2. Phenotypic Analysis of Original Leukapheresed Leukemic Cells, TMM Cells, and TMM Cells Induced to Differentiate With DMSO, TPA, and Hypoxanthine

	Original Leukapheresed Leukemic Cells	TMM Cells			
		Unstimulated	DMSO	TPA	Hypoxanthine
SmIg	−	−	−	−	−
E rosette	−	−	−	−	−
B cell	−	−	−	−	−
Fc_γ receptor	+	++	++	++	++
Leu-M1 antigen	++	++	++	++	++
CR1 (C3b/C4b receptor)	−	−	−	+	+
CR3 (C3bi receptor)	−	−	−	−	−
α-antichymotrypsin	−	−	−	−	−
Lysozyme	+	−	++	++	++
Mature myeloid cells* (%)	6	2	3	42	68

Abbreviation: SmIg, surface immunoglobulin.

*Includes myelocytes, metamyelocytes, and banded and segmented neutrophils.

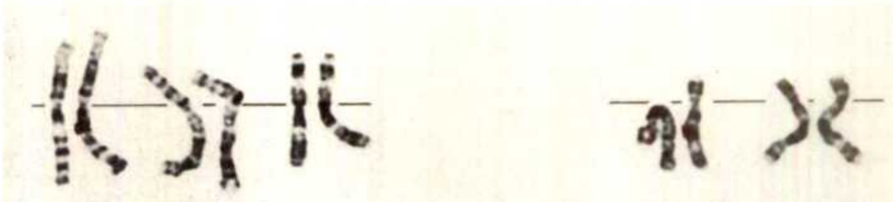

Fig 3. Giemsa-banded karyotype of the TMM line showing 46,XY.

patient's peripheral blood cells has not as yet shown any such rearrangement (Fig 4).

DISCUSSION

We herein described a novel cell line, designated TMM, derived from the peripheral blood of a 62-year-old male with a form of Ph^1-negative myeloblastic disease arising from what, in retrospect, was MDS. Several features of the patient's blood and marrow point to the blast crisis arising from an MDS, ie, thrombocytopenia with abnormal megakaryocytes, monocytosis with peripheral monocytes and promonocytes $>1 \times 10^9$/L, and abnormal erythrocytes with anemia. Therefore a current definition of the patient's disease might be MDS with chronic myleomonocytic leukemia (CMML).[6] No sideroblasts were seen, and disgranulopoiesis was not commented on in the patient's hematologic records. The relative eosinophilia is not typical and remains unexplained.

When compared, the similarity of its cellular characteristics to the original leukemic cells, and the lack of EBNA and TdT indicate that the line we have established is of blast cell origin. Cytochemical analysis showed positive, punctuate staining for acid phosphatase, and morphological studies revealed a majority of undifferentiated cells with large spherical nuclei and cytoplasm devoid of granules. A few (2%) promyelocytes, myelocytes, and metamyelocytes were also observed. The majority (90%) of the cells had a karyotype of 46,XY and expressed no chromosomal abnormalities at the light microscopic level. Restriction endonuclease digestion followed by Southern blotting showed no evidence for cryptic rearrangement of the *bcr*-c-*abl* loci. As shown in Table 2 the TMM cells expressed the myelomonocytic-specific antigen Leu-M1 as well as receptors for the Fc portion of IgG. Both myeloid and monocytoid differentiation involve expression of the Fc receptor, and therefore TMM cells were induced to differentiate to help determine their classification. Phorbol esters, purines and purine analogues, and polar planar compounds stimulate myeloid, erythroid, and monocytoid differentiation in a variety of leukemia cell lines,[23,24,33,34] and therefore TPA, hypoxanthine, and DMSO were tested on TMM cells. Induced differentiation of this line by TPA and hypoxanthine resulted in cells morphologically resembling somewhat more mature cells of the myeloid lineage and stimulated expression of lysozyme and receptors for the C3b complement protein. DMSO stimulated the expression of lysozyme only. Ultrastructural analysis of hypoxanthine-induced cells showed a higher degree of differentiation compared with unstimulated TMM cells. The induced cells were smaller and had indented to segmented nuclei and more mature cytoplasm containing many granules that stained positive for peroxidase. Such granules, reported to contain lysozyme, myeloperoxidase, and many acid hydrolases,[35,36] are important in the inflammatory, and, in associa-

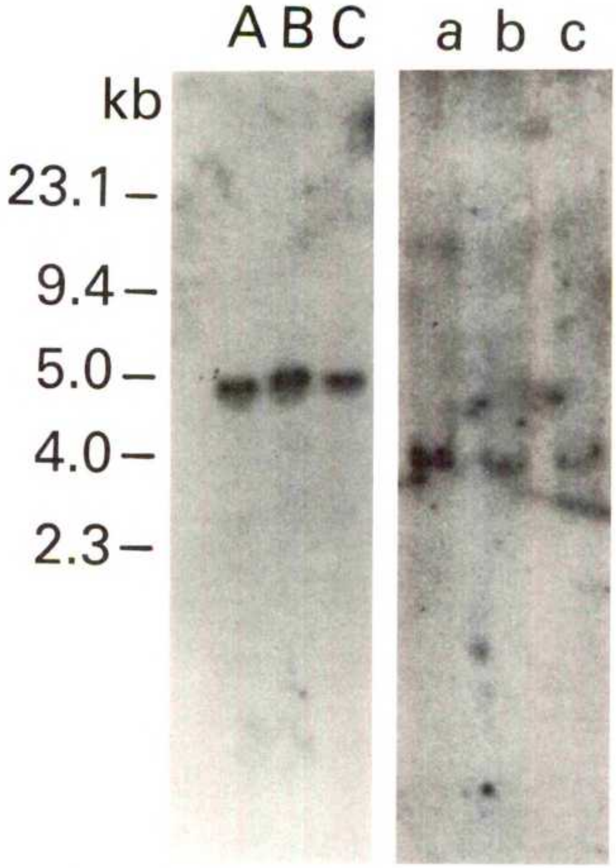

Fig 4. Southern blot of genomic DNA restricted with *BglII* and probed for *abl* and *bcr* genes. Sizes of marker DNAs in kilobases are shown at left. DNA from lanes A and a, patient's leukapheresed cells; lanes B and b, TMM cultured cells; lanes C and c, normal periperal leukocytes. Lanes A, B, and C were probed with *bcr*; lanes a, b, and c, with c-*abl*.

tion with C3b receptors, the immune response of granulocytes. The characteristics of induced TMM cells were considered to be consistent with these properties. The lack of C3bi receptors and α_1-antichymotrypsin and the inability of the cells to adhere to the growth chamber suggest that these compounds do not induce characteristics of the monocyte/macrophage lineage. TPA treatment did not induce a nonspecific esterase (α-naphthyl acetate esterase). It is unusual for phobol esters to induce differentiation toward the granulocytic lineage; however morphologically at least, that seems to have occurred in this case. The action of phorbol esters is complex; certain concentrations of TPA plus colony-stimulating factor can stimulate the proliferation of leukemic cells in vitro, and TPA can increase the number of myeloid cells per colony in tests with cells from patients with chronic myelogenous leukemia.[37-39] The biochemical markers examined thus far are consistent with the TPA induction of myeloid morphology, but of course the list of markers that can be tested is long, and leukemic cells often show irregular or inconsistent marker expression[24] so that it may be that with further testing other markers will behave differently. It is also possible that monocytoid differentiation could be induced by using other compounds.

Cell lines cultured from the blast crisis phase of various leukemias have differing properties, and these have been used to advantage in many studies. In CML, although granulocytic cells usually predominate in the chronic phase, blast crisis can result in various lineages predominating in various patients, including myeloid, lymphoid, and erythroid types.[40,41] K-562 cells, derived from a patient with CML in blast crisis, express erythroid characteristics,[42] whereas MEG-01, also established from a patient with CML in blast crisis, has megakaryocytic properties.[43] Recent evidence indicates T-cell[44] as well as B-cell involvement in blast crisis, the latter reported in the CML cell line NALM-1.[45] TMM, the new line reported here, was obtained from a patient whose clinical studies fit the criteria for MDS with CMML. TMM cells have characteristics not found in these previously established lines. TMM cells therefore may prove useful in studying the process of differentiation and proliferation of human pluripotent stem cells.

Within CML, variation in prognosis has been suggested to correlate with gene rearrangements associated with the Ph^1 chromosome. Although about 10% of CML patients lack the Ph^1 chromosome, some of these still show an associated molecular aberration involving the protooncogenes c-*sis* and c-*abl*.[46-49] C-*sis* is usually not expressed in these cells, but a c-*sis* gene product and/or platelet-derived growth factor, which share homologous sequences, may play a role in myelofibrosis, which often accompanies CML.[50,51] On the other hand, c-*abl* has been found to be rearranged to show increased expression in all cases of Ph^1-positive and in some Ph^1-negative CML.[52] An aberrant 8-kb c-*abl* mRNA transcript as well as the corresponding abnormal protein has been found in many of these patients[48,53] and in the Ph^1-positive CML cell line K-562.[54,55] Morris et al[49] have recently reported that differences in the location and expression of c-*abl* correlate with the survival heterogeneity found by Canellos et al[3] and the Rosewell Park Memorial Institute study of Ph^1-negative CML patients.[2] The value of in vitro models that would enable the expansion of our knowledge in this ill-defined category of myeloid leukemia has recently been noted.[49] It appears that patients with the normal c-*abl* gene have a much shorter survival time. Nonrandom cytogentic abnormalities have been reported in MDS. Most often these have been specific deletions or translocations, including −5, 5q−, −7, +8, 20q−, t(8:21), and +/−21.[7] Because of this, because the TMM cell line reported here came from a patient originally diagnosed as having Ph^1-negative CML, and because in any case this patient had a CMML within an MDS, the cells were tested for a cryptic rearrangement of the *bcr*-c-*abl* complex. At least one report, however, has been made of chronic MDS with the Ph^1 chromosome.[56] On the basis of our findings to date, the *bcr*-c-*abl* complex is normal in TMM cells. Again, this new cell line may be useful to workers interested in this poorly understood area of disease.

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Thrombin-Induced Changes in Platelet Membrane Glycoproteins Ib, IX, and IIb-IIIa Complex

By Alan D. Michelson and Marc R. Barnard

Platelet membrane glycoprotein Ib (GPIb) and the GPIIb-IIIa complex have central roles in the interaction of platelets with the plasma coagulation system, damaged vessel walls, and other platelets. We investigated the effects of thrombin on these glycoproteins. Monoclonal antibodies were used to assess platelet surface glycoproteins by flow cytometry, total platelet glycoprotein content by immunoassay, and glycoproteins released from platelets, also by immunoassay. Five new observations were made with regard to thrombin-induced changes in platelet membrane glycoproteins: (*a*) The marked decrease in platelet surface binding of antibodies directed at GPIb was not confined to antibodies directed at the von Willebrand factor binding site. (*b*) There was a marked decrease in platelet surface binding of an antibody directed at GPIX, with maintenance of the 1:1 ratio of platelet surface binding of antibodies directed at GPIb and GPIX. (*c*) Changes in platelet surface binding of antibodies were not restricted to a distinct subpopulation of platelets. (*d*) There was no associated platelet release of glycocalicin (a proteolytic fragment of GPIb). (*e*) There was no associated platelet release of the GPIIb-IIIa complex. These thrombin-induced changes may be important in modulating the reactivity of platelets with the damaged vessel wall and with each other.

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PLATELET MEMBRANE GLYCOPROTEIN (GP)Ib and the GPIIb-IIIa complex have central roles in the interaction of platelets with the plasma coagulation system, with damaged blood vessel walls, and with other platelets.[1] First, the glycocalicin portion of the α chain of GPIb contains a binding site(s) for thrombin.[2,3] Second, platelet adhesion to damaged blood vessel walls is mediated by the binding of the adhesive glycoprotein von Willebrand factor (vWf) to the glycocalicin portion of the α chain of platelet membrane GPIb.[1,4,5] Third, platelet aggregation is mediated by the activation-dependent binding of several adhesive glycoproteins, including fibrinogen, fibronectin, and vWf, to the platelet membrane GPIIb-IIIa complex.[1,6-8]

Thrombin-induced changes in GPIb and the GPIIb-IIIa complex may be important in modulating the reactivity of platelets with the damaged vessel wall and with other platelets. George et al[9] recently demonstrated that thrombin activation of platelets results in decreased binding of a monoclonal antibody directed at the vWf binding site on GPIb. A number of investigators have observed a thrombin-induced increase in the platelet surface expression of the GPIIb-IIIa complex.[9-12]

In unactivated platelets, platelet membrane GPIX is bound to platelet surface GPIb in a 1:1 ratio (the GPIb-IX complex).[13,14] However, the function of GPIX is unknown. In addition to their platelet surface pools, GPIb and the GPIIb-IIIa complex are present in intraplatelet pools.[10,11,15]

In the present study, a library of monoclonal antibodies was used to investigate thrombin-induced changes in GPIb, GPIX, and the GPIIb-IIIa complex. Different pools of these glycoproteins were assessed by the following techniques: (*a*) platelet surface glycoproteins by flow cytometry,[16,17] (*b*) glycoproteins released from platelets by enzyme-linked immunosorbent assay (ELISA),[5,18] and (*c*) total platelet glycoprotein content by ELISA of Triton X-100 platelet lysates.

MATERIALS AND METHODS

Antibodies. Table 1 lists the eight murine monoclonal antibodies used in this study. We used three different antibodies (6D1, WM23, and 3G6) directed against the glycocalicin portion of the α chain of platelet membrane GPIb. Antibody 6D1 is directed at or very near the vWf binding site on GPIb,[4] whereas WM23 and 3G6 are not directed against the vWf binding site (Berndt et al[13] and B. Adelman, personal communication). WM23 has been characterized as being directed against the macroglycopeptide portion of glycocalicin rather than the terminal protein portion that contains the vWf and 6D1 binding sites (M.C. Berndt, personal communication). After complete platelet solubilization with sodium dodecyl sulfate, polyacrylamide gel electrophoresis and Western blotting, both 3G6 (B. Adelman, personal communication) and WM23 (M.C. Berndt, personal communication) recognize only a single band (GPIb) in both reduced and unreduced gels. FMC25 is directed against platelet membrane GPIX.[13,19] Antibody 10E5 is directed against the platelet membrane GPIIb-IIIa complex.[20] WM18 is also directed against the platelet membrane GPIIb-IIIa complex but at a different epitope to 10E5 because WM18 has little effect on platelet aggregation (M.C. Berndt, personal communication), whereas 10E5 inhibits platelet aggregation induced by adenosine diphosphate (ADP), epinephrine, and collagen.[20] As a marker of platelet activation, we used KC4, a monoclonal antibody directed against the platelet activation–dependent granule–external membrane (PADGEM) protein.[21,22] PADGEM protein is a component of the α granule membrane of resting platelets that is expressed on the platelet plasma membrane during platelet activation.[22] PADGEM protein is distinct from the surface glycoproteins of resting platelets but identical to GMP-140, the antigen recognized by monoclonal antibody S12.[22,23] As a control in the flow cytometric assay, we used OX6, a monoclonal antibody directed against rat lymphocyte Ia antigen.[24]

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Table 1. Monoclonal Antibodies Used in This Study

Antibody	Antigen	Source*
6D1	Human platelet membrane GPIb (vWf binding site on glycocalicin)	Dr Barry Coller
WM23	Human platelet membrane GPIb (macroglycopeptide portion of glycocalicin; not the vWf binding site)	Dr Michael Berndt
3G6	Human platelet membrane GPIb (glycocalicin; not the vWf binding site)	Dr Burt Adelman
FMC25	Human platelet membrane GPIX	Dr Michael Berndt
10E5	Human platelet membrane GPIIb-IIIa complex (inhibits platelet aggregation induced by ADP, epinephrine, collagen)	Dr Barry Coller
WM18	Human platelet membrane GPIIb-IIIa complex (little effect on platelet aggregation)	Dr Michael Berndt
KC4	Human PADGEM protein	Dr Bruce Furie
OX6	Rat lymphocyte Ia antigen	Dr Bruce Woda

*For references, see Materials and Methods.

Thrombin activation of washed platelets. Blood was drawn by venipuncture from healthy adult volunteers who had not ingested aspirin within the previous ten days. The blood was drawn directly into a syringe containing $1/7$ vol of acid-citrate-dextrose (85 mmol/L trisodium citrate, 71 mmol/L citric acid, 111 mmol/L dextrose, pH 4.5) and 50 ng/mL (final concentration) prostaglandin E_1 (PGE_1, Sigma Chemical Co, St Louis). After resting for one hour at 22°C, the blood was centrifuged at 150 g for 15 minutes at 22°C and the platelet-rich plasma removed. The platelets were washed twice by centrifugation at 2,000 g for ten minutes at 22°C and resuspension in Tyrode's buffer (138 mmol/L NaCl, 29 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.4 mmol/L $NaHPO_4$, 0.1% glucose, 0.35% bovine serum albumin, pH 6.5) with 50 ng/mL PGE_1. After the second wash, 5×10^5 platelets/μL were suspended in Tyrode's buffer, pH 7.3, with 50 ng/mL PGE_1 and either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. Purified human α-thrombin (generously provided by Dr John Fenton II, New York Department of Health, Albany) at concentrations between 6.4×10^{-5}, and 1.0 U/10^8 platelets was added to the sample, which was then gently inverted once. In control assays performed in parallel, buffer only (no thrombin) was added. The samples were incubated undisturbed (to prevent platelet aggregation) for ten minutes at 37°C. To stop the reaction, hirudin (Sigma), 2.0 U/10^8 platelets, was added to all samples, including those with no thrombin. After gentle inversion, the samples were incubated at 22°C for five minutes. Each sample was then divided into two separate aliquots. The first aliquot was fixed at 22°C for 30 minutes with an equal volume of 2% formaldehyde in Tyrode's buffer, pH 7.3, with either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. These samples were then washed twice in Tris-buffered saline (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.8) by centrifugation at 2,000 g for ten minutes at 22°C, resuspended at 50,000 platelets/μL in Tris-buffered saline, and assayed by flow cytometry for platelet surface glycoproteins (see the next section). The second aliquot of thrombin-activated and control platelets was centrifuged at 2,000 g for ten minutes at 22°C. The supernatants were removed for ELISA determination of platelet release of glycocalicin (a proteolytic fragment of GPIb) and the GPIIb-IIIa complex (see later). The platelet pellets were lysed in Tyrode's buffer containing 1% Triton X-100 (Sigma), 50ng/mL PGE_1, and either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. Before being assayed, the supernatants and lysates were stored at −80°C for up to seven days. Before assay by ELISA for the total platelet content of GPIb and the GPIIb-IIIa complex (see later), the lysates were thawed and, to remove any cellular debris, centrifuged at 22°C for four minutes at 8,000 g (conditions that result in only a small degree of sedimentation of actin filament–associated GPIb[25]).

Flow cytometric assessment of platelet surface glycoproteins. Fixed, washed thrombin-activated and control platelets (50,000/μL) were prepared as described earlier and incubated for 20 minutes at 22°C with a saturating concentration of one of five monoclonal antibodies (6D1, WM23, 3G6, FMC25, or 10E5) to assess platelet surface glycoproteins. In parallel experiments, aliquots of these platelet suspensions were incubated with either monoclonal antibody KC4 or monoclonal antibody OX6 to assess platelet activation and background binding, respectively. As described by George et al[9] the platelet surface GPIIb-IIIa complex was assessed on platelets activated in a Ca^{++}-containing buffer, whereas the platelet surface GPIb-IX complex was assessed on platelets activated in an EDTA-containing buffer. Thus, the platelet samples that had been prepared in the presence of 2 mmol/L $CaCl_2$ (see earlier) were incubated with either 10E5 or OX6, whereas the platelet samples that had been prepared in the presence of 5 mmol/L EDTA were incubated with either 6D1, WM23, 3G6, FMC25, or OX6. Platelet surface expression of PADGEM protein was assessed by antibody KC4 on platelets prepared in the Ca^{++}-containing buffer. All platelet samples were then washed twice in Tris-buffered saline by centrifugation at 2,000 g for ten minutes at 22°C and incubated for 20 minutes at 22°C with a saturating concentration of fluorescein isothiocyanate—labeled goat antimouse IgG antibody (Cooper Biomedical, Malvern, PA). The platelets were then washed twice, resuspended in Tris-buffered saline, and analyzed in a FACS 440 (Becton Dickinson, Mountain View, CA). For each sample, the fluorescence signal from 10,000 individual cells was measured. Forward-light scatter profiles indicated that there was neither platelet clumping nor detectable contamination by other cell types in any of the samples. Histograms were gated for platelets by their characteristic forward and orthogonal light scatter, thereby excluding any possible contribution from other cells or debris. To compare results in linear form, data obtained from fluorescence channels in a logarithmic mode were converted to their linear equivalents (relative specific fluorescence) as previously described.[17]

ELISA determination of platelet release of glycocalicin and the GPIIb-IIIa complex. Supernatants obtained from centrifugation of thrombin-activated and control platelets were assessed for their glycocalicin content by an ELISA using monoclonal antibody 6D1 as previously described.[5,18] In parallel assays, 6D1 was replaced by either monoclonal antibody 10E5 or monoclonal antibody WM18. As described earlier, the supernatants used for assays with 6D1 were EDTA-containing, whereas the supernatants used for assays with 10E5 and WM18 were Ca^{++}-containing.

ELISA determination of total platelet content of GPIb and the GPIIb-IIIa complex. Lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were assessed in the ELISA for their ability to inhibit monoclonal antibody 6D1 binding to platelet surface GPIb. This assay measures the total number of GPIb receptors for vWf because 6D1 binds with equal avidity to GPIb and glycocalicin.[5] The assay was validated by demonstrating a comparable total number of GPIb molecules per platelet in experiments in which the monoclonal antibody to GPIb was changed from 6D1 to WM23 or 3G6 and in other experiments in which, rather than Triton X-100 platelet lysates, the aqueous phase of Triton X-114–solubilized platelets[26] was assessed (A.D. Michelson, M.R. Barnard, E. Carroll: unpublished observations). Serial dilutions of the lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were also assessed in the ELISA for their ability to inhibit the binding of either monoclonal antibody 10E5 or monoclonal antibody WM18 to the platelet surface. As described earlier, the lysates used for assays with 6D1 were EDTA-containing, whereas the lysates used for assays with 10E5 and WM18 were Ca^{++}-containing. Control assays in which platelets were replaced by buffer only and then treated with 1% Triton X-100 resulted in the detection of no GPIb ($n = 3$) or GPIIb-IIIa complex ($n = 3$), thereby demonstrating that the dilutions of Triton used did not interfere with the ELISA.

Platelet counts. Platelet counting was performed on a Coulter Model ZBI (Coulter Electronics, Hialeah, FL).

RESULTS

A flow cytometric method was used to demonstrate the effect of thrombin on platelet surface glycoproteins. As shown in Fig 1, thrombin resulted in a marked reduction in the platelet surface binding of each of three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on GPIb (Table 1). In addition, thrombin resulted in a similar reduction in the platelet surface binding of a monoclonal antibody (FMC25) directed against GPIX (Fig 1). In contrast, thrombin resulted in a marked increase in the platelet surface binding of a monoclonal antibody (10E5) directed against the GPIIb-IIIa complex (Fig 1). Parallel assays using monoclonal antibody KC4 (directed against PADGEM protein) demonstrated that the thrombin-induced changes in the platelet surface binding of monoclonal antibodies directed against GPIb, GPIX, and the GPIIb-IIIa complex were associated with platelet activation (Fig 1).

As determined by relative specific fluorescence, in unactivated platelets the ratio of platelet surface binding of 6D1:FMC25:10E5 (monoclonal antibodies directed at GPIb, GPIX, and the GPIIb-IIIa complex, respectively) was 1.0:0.9:2.0 (means of five separate experiments). These ratios are comparable to the ratio of platelet surface GPIb:GPIX:GPIIb-IIIa complex reported by other investigators using radioligand binding methods.[1,13,14] In maximally activated platelets (thrombin, 1.0 U/10^8 platelets), the ratio of platelet surface binding of 6D1:FMC25:10E5 was 1.0:0.9:29.1 (means of five separate experiments). Similarly, despite the thrombin-induced changes, there was maintenance of the approximately 1:1 ratio of the platelet surface binding of 6D1:FMC25 at all concentrations of thrombin tested (Figs 1 and 2).

The flow cytometric method of analyzing platelet surface glycoproteins is able to detect distinct subpopulations of platelets because each platelet is analyzed individually.[16,17] However, the thrombin-induced changes in binding of monoclonal antibodies to platelet surface GPIb, GPIX, and the GPIIb-IIIa complex were not restricted to a distinct subpopulation of platelets, irrespective of whether the thrombin concentration resulted in partial or complete activation of

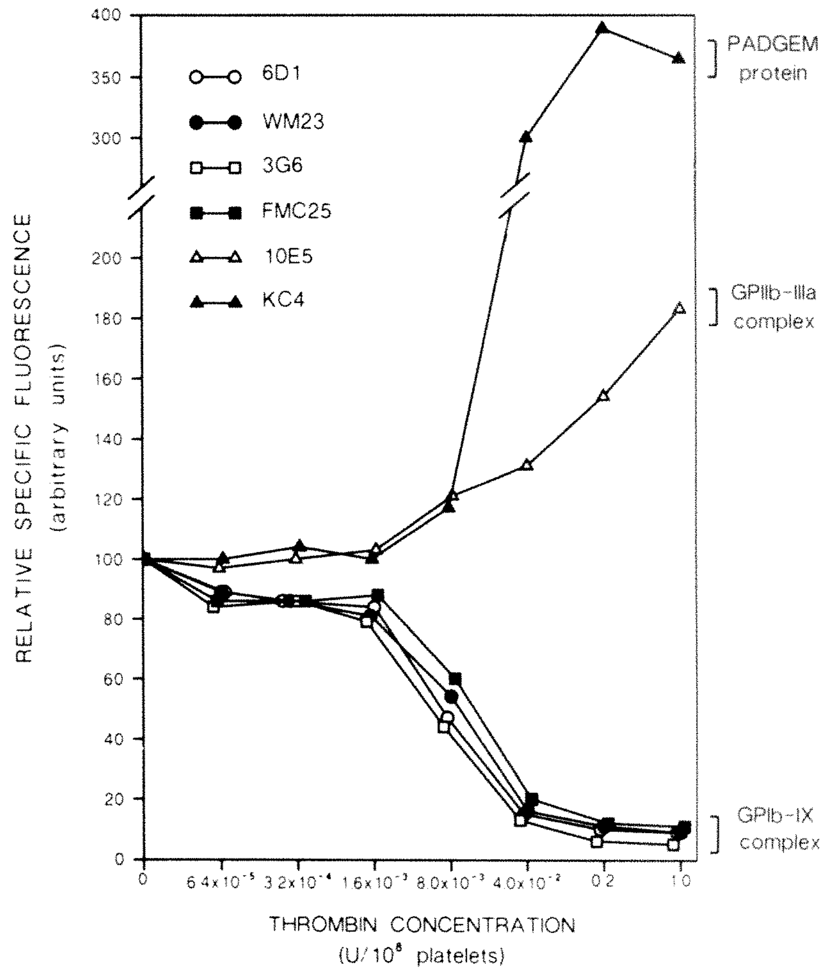

Fig 1. Effect of thrombin on the binding of monoclonal antibodies to the platelet surface as determined by flow cytometry. Platelets were incubated with monoclonal antibodies directed against either GPIb (6D1, WM23, and 3G6), GPIX (FMC25), the GPIIb-IIIa complex (10E5), or PADGEM protein (KC4). All samples were then incubated with fluoresceinated goat antimouse antibody. For each antigen, the relative specific fluorescence of control platelets was arbitrarily assigned 100 units.

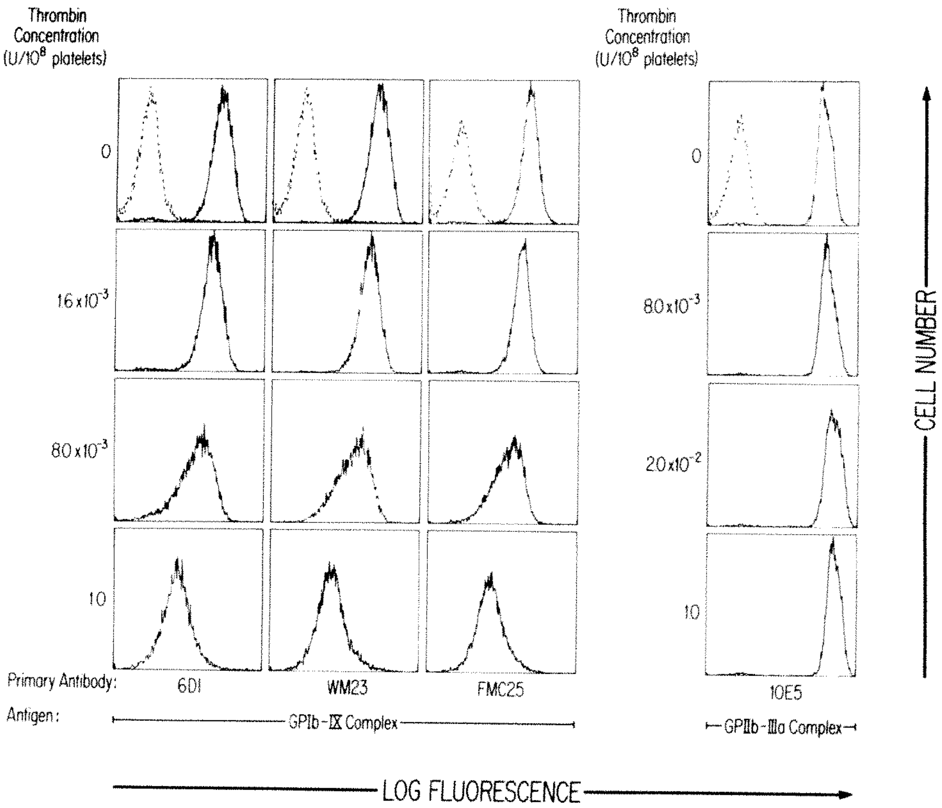

Fig 2. Effect of different concentrations of thrombin on the binding of monoclonal antibodies to the platelet surface as determined by flow cytometry. The solid lines represent platelets activated with the indicated concentrations of thrombin and then incubated with the indicated primary monoclonal antibody. The dashed lines represent platelets incubated with an irrelevant primary monoclonal antibody (background). All samples were then incubated with a fluoresceinated goat antimouse antibody. For each panel, the x-axis represents log fluorescence, and the y-axis represents cell number.

platelets (Fig 2). This is demonstrated in Fig 2 by the fact that, for all antibodies tested, increasing concentrations of thrombin resulted in a gradual shift of a single peak (to the left for the GPIb-IX complex, to the right for the GPIIb-IIIa complex).

For both control and maximally thrombin-activated platelets, the total platelet GPIb content and glycocalicin released from platelets were determined by ELISA using antibody 6D1. As shown in the upper portion of Table 2, the total platelet content of GPIb did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. As shown in the lower portion of Table 2, the glycocalicin released from platelets was, as expected, very low in the control and did not rise appreciably in the thrombin-activated platelets.

For both control and maximally thrombin-activated platelets, the total platelet content and platelet release of the GPIIb-IIIa complex were determined by ELISA using antibody 10E5. As shown in Fig 3, the total platelet content of the GPIIb-IIIa complex did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. Likewise, the platelet release of the GPIIb-IIIa complex was very low in the control and did not rise significantly in the thrombin-activated platelets (Fig 3). In parallel assays using antibody WM18, there was similarly no thrombin-induced change in the total platelet content of the GPIIb-IIIa complex and no release of the GPIIb-IIIa complex from platelets (data not shown).

Table 2. Effect of Thrombin (1.0 U/10^8 Platelets) on the Total Platelet GPIb Content and Release of Glycocalicin as Determined by ELISA Using Monoclonal Antibody 6D1

Parameter	Percentage of Total GPIb Content of Control Platelets (Mean ± SEM, n = 6)
Total platelet GPIb content*	
Control platelets	100.0 ± 18.4
Thrombin-activated platelets	97.4 ± 18.7
Glycocalicin released from platelets†	
Control platelets	0.3 ± 0.05
Thrombin-activated platelets	0.9 ± 0.1

*Determined from 1% Triton X-100 lysates of platelets.

†Determined from supernatants.

DISCUSSION

In this study, the effects of thrombin on platelet membrane GPIb, GPIX, and the GPIIb-IIIa complex were investigated. Five new observations were made.

First, the thrombin-induced change in the glycocalicin portion of the α chain of platelet surface GPIb was not confined to its vWf binding site. In addition to the previously reported[9] thrombin-induced marked decrease in binding of a monoclonal antibody directed at the vWf binding site on GPIb, we observed a comparable marked decrease in the binding of two other monoclonal antibodies directed at different epitopes on the glycocalicin portion of the α chain of GPIb. The thrombin-induced decrease in binding of monoclonal antibodies directed at GPIb was not due to occupation of the antibody binding site by thrombin bound to its receptor on GPIb[2,3] because (*a*) three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on

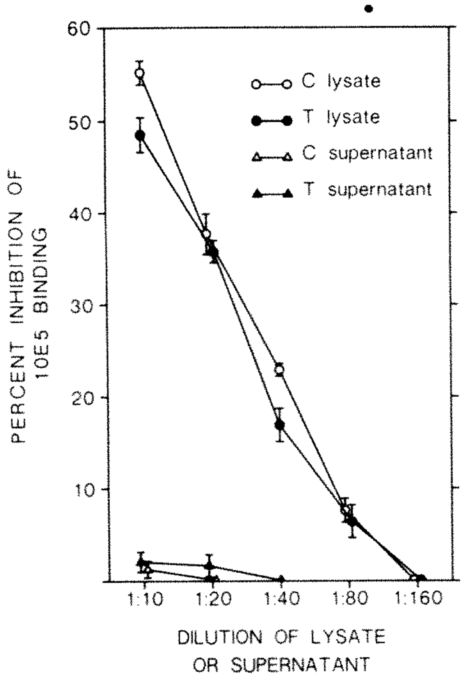

Fig 3. **Effect of thrombin (1.0 U/10^8 platelets) on the total platelet content and release of the GPIIb-IIIa complex as determined by ELISA using monoclonal antibody 10E5. The total platelet content of the GPIIb-IIIa complex was determined from 1% Triton X-100 lysates of control platelets (open circles, C lysate) and thrombin-activated platelets (closed circles, T lysate). Platelet release of the GPIIb-IIIa complex was determined from supernatants of control platelets (open triangles, C supernatant) and thrombin-activated platelets (closed triangles, T supernatant). Results are means ± SEM, n = 3.**

GPIb produced similar results and (*b*) monoclonal antibody 6D1 does not interfere with the binding of thrombin to platelets.[2]

Second, we observed a thrombin-induced marked decrease in the binding of a monoclonal antibody directed at GPIX. Although the function of GPIX is presently unknown, our data suggest the possibility that GPIX has a role in platelet activation. It has previously been demonstrated in unactivated platelets that surface GPIb is bound to GPIX in a 1:1 ratio (the GPIb-IX complex).[13,14] The present data suggest that during the thrombin-induced changes in platelet surface GPIb and GPIX there is maintenance of this 1:1 ratio.

Third, the flow cytometric method enabled us to determine that the thrombin-induced changes in the binding of monoclonal antibodies to GPIb, GPIX, and the GPIIb-IIIa complex were not restricted to a distinct subpopulation of platelets. Radioligand binding studies such as those used by George et al[9] are unable to make this distinction because the results are averaged measurements of large numbers of platelets. In contrast, because each platelet is analyzed individually, flow cytometry is able to detect distinct subpopulations of platelets, as we have recently demonstrated in children with chronic myeloid leukemia.[17]

Fourth, despite the thrombin-induced marked decrease in binding of monoclonal antibody 6D1 to platelet surface GPIb, quantitative ELISA (performed in parallel and using the same monoclonal antibody) revealed no significant thrombin-induced release of glycocalicin from the platelet and no change in the total platelet content of GPIb. In view of our previous finding that proteolytic cleavage of glycocalicin does not interfere with its measurement by our ELISA,[18] the direct measurement of released glycocalicin in the present study demonstrated that the thrombin-induced marked decrease in the platelet binding of anti-GPIb monoclonal antibodies was not the result of cleavage of platelet surface GPIb. The present study together with previous studies by other investigators[27,28] suggests that platelet surface GPIb is still present on the platelet surface after thrombin activation but its accessibility to multiple probes is greatly decreased because of either (*a*) the marked clustering of GPIb molecules that occurs on the platelet surface after thrombin activation[29]; (*b*) the marked shape change that occurs in platelets during thrombin activation (less likely because of the associated increased accessibility of surface GPIIb-IIIa complex); or (*c*) a major conformational change in the GPIb-IX complex (also less likely because such a change would not be expected to result in the same decrease in binding of four monoclonal antibodies [6D1, WM23, 3G6, and FMC25] directed at different epitopes on the GPIb-IX complex and three of these antibodies [WM23, 3G6, and FMC25] recognize their epitope on Western blots, which suggests that they are relatively insensitive to conformational changes in the antigen).

Fifth, unlike α granule constituents such as platelet factor 4 and β-thromboglobulin,[30] the GPIIb-IIIa complex was not released from platelets during thrombin activation. Although the present investigation did not directly exclude the possibility that uncomplexed GPIIb and/or GPIIIa subunits were released from platelets during thrombin activation, the lack of change in the total platelet content of the GPIIb-IIIa complex, as determined by two different complex-specific monoclonal antibodies, did not support this view.

In conclusion, given the central roles of GPIb and the GPIIb-IIIa complex in platelet adhesion and aggregation,[1] the presently described thrombin-induced changes in these glycoproteins are likely to be important in modulating the reactivity of platelets with the damaged blood vessel wall and with other platelets.

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Localization of a Factor VIII Binding Domain on a 34 Kilodalton Fragment of the *N*-Terminal Portion of von Willebrand Factor

By Yukihiro Takahashi, Michel Kalafatis, Jean-Pierre Girma, Karin Sewerin, Lars-Olov Andersson, and Dominique Meyer

Factor VIII (F.VIII) was tested for its ability to bind in solid phase system to von Willebrand Factor (vWF) or fragments obtained with *Staphylococus aureus* V-8 protease, ie, SpIII (*N*-terminal), SpI (central), and SpII (C-terminal). Bound F.VIII was estimated in situ by clotting and chromogenic assays. F.VIII bound in a dose-dependent manner to immobilized vWF and SpIII but not to SpII or SpI. Binding was inhibited by 0.25 mol/L $CaCl_2$ as well as by an excess of vWF or SpIII. Accordingly, immobilized F.VIII specifically bound ^{125}I-vWF and SpIII but not SpII or SpI. Twelve monoclonal antibodies (MoAbs) directed towards SpIII, specifically blocking binding of F.VIII to vWF or SpIII, were used for the mapping of plasmic or tryptic fragments of vWF or SpIII. We thus established that a F.VIII binding domain of vWF is located on a 34 kilodalton (kd) fragment of the *N*-terminal portion of vWF, between residues 1 and 910, and that it is distinct from the GPIb and collagen binding domains.

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HUMAN von Willebrand Factor (vWF) mediates adhesion of platelets to the subendothelium of the vessel wall and serves as Factor VIII (F.VIII) carrier, stabilizing its activity.[1] Results of proteolytic degration of vWF[2-13] have shown that the basic 270 kilodalton (kd)-subunit of vWF contains distinct functional domains. *Staphylococus aureus* V-8 protease (V-8 protease) hydrolyses vWF-subunit at Glu 1,365 producing two fragments, SpII, a dimer of the C-terminal portion (residues 1,366 to 2,050) containing a binding domain for GPIIb/IIIa[5] and SpIII, a dimer of the *N*-terminal portion of vWF (residues 1 to 1,365). SpIII contains binding domains for GPIb[4-6] and for heparin,[13] both being localized between residues 449 and 728 [8,13]; and two distinct domains interacting with collagen (residues 542 to 622 and 948 to 998).[9-12] The latter is in the central part of vWF-subunit fragment SpI, (residues 911 to 1,365) obtained by secondary digestion of SpIII with V-8 protease.[11,12] The localization of the domain of vWF that binds to F.VIII is unknown. Using fragments of vWF obtained with V-8 protease we demonstrate here that a F.VIII binding domain is located on a 34 kd species of the *N*-terminal part of SpIII, between amino acid residues 1 and 910 of vWF-subunit.

MATERIALS AND METHODS

Purification of human vWF and fragmentation. Human vWF was purified according to Thorell and Blomback.[14] It was digested with V-8 protease (Miles Laboratories, Inc, Naperville, IL) and proteolytic fragments SpI, SpII and SpIII were purified as reported.[4,5] Purified vWF or SpIII were digested for 24 hours in 25 mmol/L Tris-HCl, pH 8, 0.15 mol/L NaCl with Trypsin-TPCK[14] (Worthington, Freehold, NJ) at 22°C, or with plasmin[15] at 37°C. Plasmin was produced by incubating human Glu-plasminogen (gift of Dr E. Anglés-Cano, INSERM, U. 143, Paris) for one hour at 37°C with 10 IU/mL of human urokinase (gift of Dr F. Toulemonde, Institut Choay, Paris).

Purification of human F.VIII. Human F.VIII was purified from cryoprecipitate according to Andersson et al.[16] The preparation contained ~100 U/mL of F.VIII (~7,000 U/mg) and <0.001 U/mL of vWFAg.

Antibodies to vWF or to F.VIII. Among 28 monoclonal antibodies (MoAbs) to vWF reacting with SpIII, three are already known to inhibit the binding of vWF or SpIII to GPIb (MoAbs 311, 322, 328) and six, also directed to SpI, to block the binding of vWF or SpIII to collagen (MoAbs B200 to B205).[5,11,12] Among ten MoAbs to vWF directed against SpII, one (MoAb 9) is known to inhibit the binding of vWF or SpII to GPIIb/IIIa.[5] Two antibodies (Abs) to F.VIII were used: MoAb D_4H_1, with an anti-VIIIC titer of 5 Bethesda U/mg IgG and an oligoclonal IgG purified from the plasma of a hemophilia A patient with a high titer (1,500 U/mL) inhibitor.

Radiolabeling. Proteins were labeled with ^{125}I as described.[5] Specific radioactivity of vWF, SpIII, SpII, or SpI was 0.9, 2.5, 4.2, and 1.3 μCi/μg, respectively.

Binding of F.VIII to vWF or vWF proteolytic fragments. A solid phase binding assay was performed in 3 mL-polystyrene tubes, using a final volume of 0.5 mL at each step. vWF, SpIII, SpII, or SpI (4 μg/mL in 0.05 mol/L sodium carbonate, pH 9.8) were coated for three hours at 37°C. Following washing with 25 mmol/L sodium barbital-acetate buffer, pH 7.3 (Michaelis buffer) containing 0.3% BSA and 0.05% Tween 20 (washing buffer), potential traces of F.VIII were eliminated by incubating for 30 minutes at 37°C with 0.25 mol/L $CaCl_2$ in Michaelis buffer and washing. F.VIII (0 to 2 U/mL in 3% BSA-Michaelis buffer) was then incubated for one hour at 37°C. Unbound F.VIII was eliminated by washing and bound F.VIII was estimated in situ. Nonspecific binding of F.VIII was estimated using fibrinogen, fibronectin, or albumin-coated tubes. In control experiments, selected MoAbs (IgG) were previously coated to the tubes at a concentration of 20 μg/mL and vWF or fragments were immobilized as already described. In both systems, the amount of immobilized vWF or fragments was determined at various steps of the assay by either mixing ^{125}I-vWF or fragments with the unlabeled protein or by reacting for three hours at 37°C the content of the tubes with a specific ^{125}I-MoAb IgG to SpI, SpII, or SpIII in 3% BSA-Michaelis buffer, followed by washing and counting. Inhibition of F.VIII binding was tested by incubating 2 U/mL of F.VIII premixed with increasing amounts (0 to 10 μg/mL) of vWF, SpI, SpII, or SpIII as competitor. MoAbs (IgG) to vWF were used at a concentration of 20 μg/mL.

Assay of F.VIII. Bound F.VIII was estimated in situ using a one-stage activated partial thromboplastin time by adding to the tubes 100 μL of Michaelis buffer, 100 μL of F.VIII deficient plasma (Diagnostica Stago, Asnières, France), 100 μL of 5% kaolin and 100 μL of cephalin. Following three minutes at 37°C, coagulation was initiated by 100 μL of 25 mmol/L $CaCl_2$. A chromogenic assay

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(Coatest factor VIII, KabiVitrum, Stockholm) was performed as described by the manufacturer except that 300 μL of Michaelis buffer were added before 100 μL of the other reagents. Results were compared with those using 0 to 10 μL of normal pool plasma (1 U/mL of F.VIII) in 100 μL of buffer for the clotting assay and in 300 μL for the chromogenic assay.

Binding of vWF or vWF degradation fragments to F.VIII. Following precoating of MoAb D4H1 (IgG) at a final concentration of 60 μg/mL, washing and incubation of F.VIII (8 U/mL) for two hours at 37°C, serial dilutions of ^{125}I-vWF, SpI, SpII, or SpIII in 3% BSA-Michaelis buffer were incubated in the tubes for one hour at 37°C and 18 hours at 4°C. Following washing, the bound material was estimated by counting. Nonspecific binding was determined in the absence of F.VIII.

Gel analysis. SDS-polyacrylamide gel electrophoresis was performed and gels were stained with coomassie blue or transferred to nitrocellulose, reacted with selected ^{125}I-antibody IgG and autoradiographed as described.[5]

RESULTS

Binding of F.VIII to immobilized vWF or vWF fragments. The amount of coated vWF or SpI, SpII, or SpIII was consistent, dose-dependent, and saturable, reaching a maximum of ~500 ng of protein bound per tube out of 2 μg applied in 500 μL. Under these conditions, purified F.VIII specifically bound to coated vWF and to SpIII in a dose-dependent and saturable manner (Fig 1) with a maximum reached at ~0.5 U/tube of F.VIII. Comparison of these results with the control curve obtained using serial dilutions of normal pool plasma in uncoated tubes (Fig 1, insets) showed that a maximum of ~0.01 U of purified F.VIII was bound per coated tube. F.VIII was unable to bind to immobilized SpII or SpI as well as to BSA, fibrinogen or fibronectin (Fig 1). Control experiments showed that bound F.VIII could be totally removed by incubating with 0.25 mol/L $CaCl_2$. Bound F. VIII was markedly inhibited by MoAb to F.VIII D4H1 at concentrations ≥ 30 μg/mL and completely neutralized by the oligoclonal Ab to F.VIII (results not shown). Measurement of vWF or fragments using ^{125}I-MoAbs 9 or B203 indicated that the amount of coated protein was constant throughout the various steps of the assay. Thus the increase of clotting times was not related to a loss of coated protein. In addition, specific binding of F.VIII to vWF or to SpIII and absence of binding to SpII or to SpI was observed when the proteins were immobilized through a MoAb to vWF coated to the tube. Specificity of F.VIII binding to vWF or to SpIII was confirmed in experiments of competitive inhibition (Fig 2). Results were similar whether the substrate was coated vWF (Fig 2A) or SpIII (Fig 2B). By contrast SpII or SpI had no significant effect on F. VIII binding even at a concentration of 10μg/mL.

Binding of ^{125}I-vWF or vWF fragments to antibody-bound

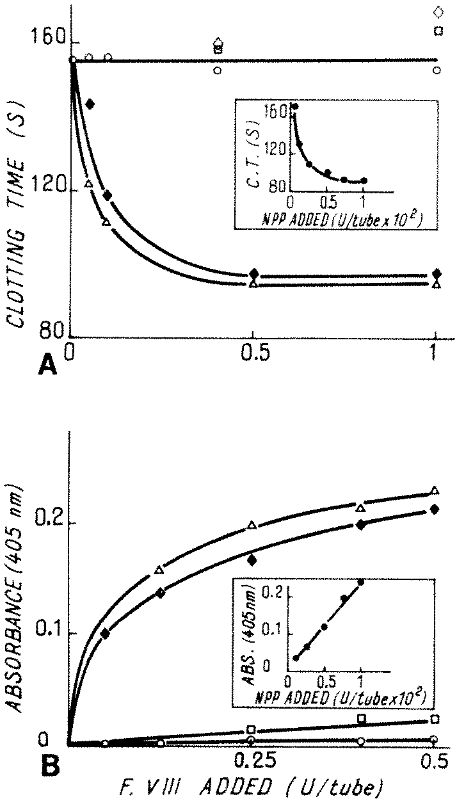

Fig 1. Binding of purified F.VIII to immobilized vWF or vWF degradation fragments. Bound F.VIII was estimated in situ using either a one-stage clotting method (A) or a chromogenic assay (B) as described in Materials and Methods. Binding of F.VIII was estimated using tubes coated with BSA (◇), SpI (□), SpII (○), SpIII (♦), or vWF (△). Insets show the control dose-response curves obtained using various amounts of normal pool plasma (NPP) in uncoated tubes.

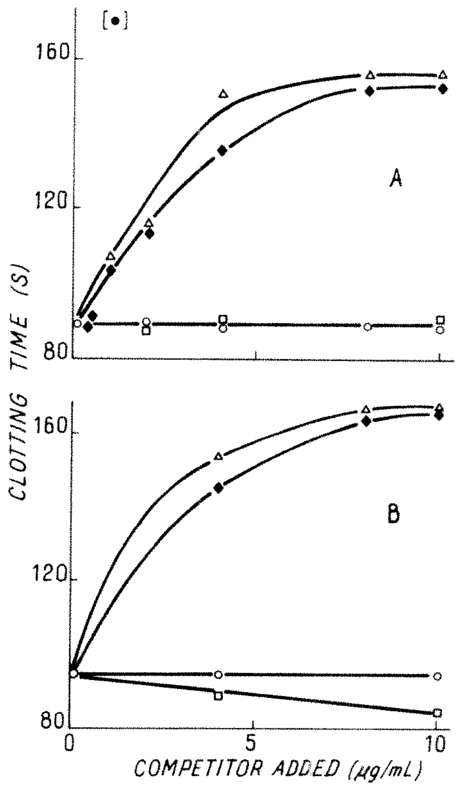

Fig 2. Competitive inhibition of F.VIII binding to vWF- (A) or SpIII-coated tubes (B) by increasing concentrations of competitor vWF (△), SpI (□), SpII (○), or SpIII (♦). Bound F.VIII was estimated by its clotting activity. [●] indicates the control clotting time when using BSA-coated tubes.

F.VIII. Using F.VIII immobilized onto antibody coated tubes, a dose-dependent binding of ^{125}I-SpIII or ^{125}I-vWF was observed, eight and 35 times higher than the control, respectively. ^{125}I-SpII or ^{125}I-SpI did not bind to F.VIII under the same conditions (not shown).

Effect of MoAbs to vWF on binding of F.VIII to coated vWF or SpIII. Among 28 MoAbs reacting with SpIII, 12 MoAbs derived from three fusions (MoAbs 418, 424, 426, 440, 456, 464, 468, 469, 470, 477, 479, 480) almost completely blocked binding of F.VIII to coated vWF or SpIII (95% to 98% inhibition). They did not bind to SpI but recognized a small number of intermediates among those produced during the limited digestion of vWF and of SpIII with trypsin or plasmin (Fig 3). Kinetic studies of the digestion established that the 34 kd fragment consistently observed (Fig 3) derived from the larger immunoreactive fragments and represented the smallest species immunodetectable (not shown). The other MoAbs to SpIII as well as MoAbs to SpII had no inhibitory effect on F. VIII binding to vWF or SpIII (not shown).

DISCUSSION

The location on vWF of the domain specific for F.VIII is unknown. In this study we estimated in a solid phase system the interaction between purified F.VIII and vWF or its fragments produced by V-8 protease (SpI, SpII, and SpIII).[4,5] Our data demonstrate that a vWF-domain interacting with F.VIII is located on the *N*-terminal part of the subunit. Our findings also strongly suggest that there is no other F.VIII binding domain on the rest of the molecule. We have thus established that F.VIII binds in a specific and dose-dependent manner to immobilized vWF and SpIII, a dimer of the *N*-terminal portion of vWF-subunit (residues 1 to 1,365). Conversely, F.VIII does not bind to SpII, a dimer of the C-terminal end of vWF-subunit (residues 1,366 to 2,050) nor to SpI, the C-terminal part of SpIII (residues 911 to 1,365). Thus, the binding domain for F.VIII on the vWF-subunit is localized in the *N*-terminal part of SpIII, between residues 1 and 910. Control experiments in which accessibility to the bound protein was modified by using fragments immobilized on previously coated MoAbs indicated that the absence of a binding site for F.VIII on SpI and SpII was not related to steric hindrance. In addition inhibition experiments of F.VIII binding to coated vWF or SpIII by vWF, SpI, SpII, or SpIII clearly confirmed that solely SpIII was able to substitute for vWF and interact with F.VIII. Similarly, when F.VIII was immobilized through a specific MoAb, significant binding of labeled vWF and SpIII was observed, contrasting with a total lack of binding of SpII.

Additional evidence for the presence of a F.VIII binding domain at the *N*-terminal part of SpIII was obtained using MoAbs to different regions of the vWF-subunit. Thus, the absence of noticeable effect on F.VIII binding to vWF or SpIII by MoAbs previously known to block the interaction of vWF with GPIb and collagen[5] strongly supports that the F.VIII binding domain is distinct from those involved in platelet adhesion to the subendothelium. In addition the complete inhibition of binding induced by a series of MoAbs to vWF also reacting with SpIII but not with SpI confirms the presence of a F.VIII binding domain on a portion extending between amino acid residues 1 and 910. We observed that these MoAbs recognize a 34 kd fragment produced in the early phase of digestion of vWF and SpIII by trypsin or plasmin. Recent data from Hamilton et al[15] demonstrated that a 34 kd fragment containing that *N*-terminal sequence of vWF-subunit was produced by early cleavage with these enzymes. We thus assume that the F.VIII binding domain of vWF is located on the previously identified 34 kd *N*-terminal fragment.

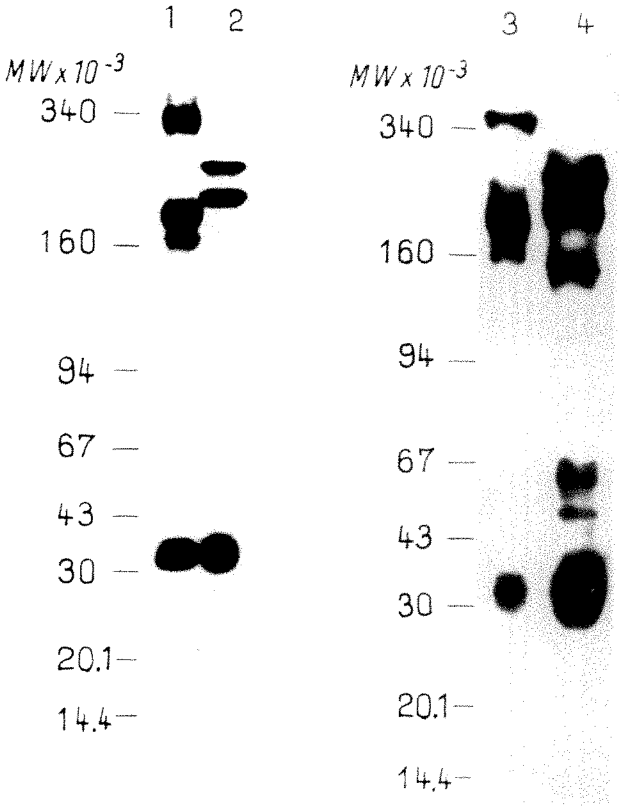

Fig 3. Immunostaining with MoAb to SpIII 418 of the intermediate products of the limited digestion of vWF or SpIII with trypsin at 22°C for 24 hours using an enzyme-substrate ratio of 1:1000 (lanes 1, 2) or with plasmin at 37°C for 24 hours using a ratio of 1:50 (lanes 3, 4). Following electrophoresis in SDS-polyacrylamide (3.5% to 20% gradient), transfer onto nitrocellulose paper and incubation with the ^{125}I-MoAb, the proteins were displayed by autoradiography. Position of the mol wt markers (fibrinogen: 340 kd; IgG: 160 kd and commercial mol wt markers) is indicated on the left.

NOTE ADDED IN PROOF

Subsequent to the submission of this manuscript to BLOOD, the paper "A Major Factor VIII Binding Domain Resides within the Amino-Terminal 272 Amino Acid Residues of von Willebrand Factor" by P.A. Foster, C.A. Fulcher, T. Marti, K. Titani, and T.S. Zimmerman appeared in the *Journal of Biological Chemistry*, 262:8443, 1987.

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CONCISE REPORT

Calcium-Dependent Cysteine Protease Activity in the Sera of Patients With Thrombotic Thrombocytopenic Purpura

By William G. Murphy, Jane C. Moore, and John G. Kelton

Plasma and serum from patients with thrombotic thrombocytopenic purpura (TTP) can cause activation and aggregation of normal human platelets in vitro. It is possible that this platelet-activating factor contributes to the disease. In this report we describe studies designed to identify the platelet-activating factor in TTP. Platelet activation by sera from 15 patients with TTP was inhibited by leupeptin, iodoacetamide, and antipain but not by phenylmethylsulphonylfluoride, ε-aminocaproic acid, soybean trypsin inhibitor, aprotinin, and D-phenylanyl-1-prolyl-1-arginine chloromethyl ketone. These studies suggested that the platelet-activating factor in TTP serum was a cysteine protease. We confirmed that a calcium-dependent cysteine protease (CDP) was present in the sera of each of the 15 patients when we used an assay based on the ability of CDP to proteolyse platelet membrane glycoprotein 1b (GP1b) and hence to abolish the ability of CDP-treated normal platelets to agglutinate in the presence of ristocetin and von Willebrand factor. This proteolytic activity was inhibited by EDTA, leupeptin, antipain, iodoacetamide, and by *N*-ethylmaleamide (NEM) but not by the serine protease inhibitors. Activity was detected in 15 of 15 patients with TTP tested before therapy was begun. In contrast, no activity was detected in the serum of any of five of the TTP patients tested in remission or in any of the sera from 36 patients with thrombocytopenia and 423 nonthrombocytopenic controls. To look for in vivo CDP activity in patients with TTP, we studied platelets from two patients with acute TTP (drawn into acid-citrate-dextrose, NEM, and leupeptin). These platelets showed a loss of GP1b from the platelet surface. Both patients were also studied in remission: GP1b on the platelet surface had returned to normal. These studies provide evidence that CDP is present in the sera of patients with TTP, that it is specific to this disease, and that it is active in vivo as well as in vitro. We postulate that a disorder of CDP homeostasis plays a major role in the pathophysiology of TTP.

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IT HAS BEEN SHOWN that the plasma[1] and serum[2] of patients with thrombotic thrombocytopenic purpura (TTP) contain a factor that causes activation and aggregation of normal human platelets in vitro; it is possible that this factor causes the platelet thrombi in the microvasculature that are the characteristic pathological lesions of the disease.[3] In this report we describe further studies that were designed to identify the platelet-activating factor in the serum of patients with TTP. The studies were in three parts: (*a*) Studies of the effects of enzyme inhibitors on platelet 14C-serotonin release. TTP and control sera were incubated with a panel of protease inhibitors and then added to ^{14}C-serotonin–labeled normal platelets. The release of ^{14}C-serotonin from the labeled platelets was measured. Because cysteine protease inhibitors blocked the platelet activation caused by the TTP sera, we postulated that the platelet-activating factor of TTP was a calcium-dependent cysteine protease (CDP).

(*b*) Part 2 dealt with confirmation of the presence of CDP activity in the sera of patients with TTP. To confirm that CDP was present in the sera of patients with TTP, we tested the effect of the sera on platelet membrane glycoprotein Ib (GPIb): CDP cleaves glycocalicin from GPIb.[4] The loss of GPIb results in the inability of the platelets to agglutinate in the presence of ristocetin and von Willebrand factor (vWF). After incubation of formalin-fixed normal platelets with TTP sera in the presence and absence of protease inhibitors, we studied whether the platelets could agglutinate in the presence of ristocetin and normal plasma. Sera from 15 patients with acute TTP caused normal fixed platelets to lose their ability to agglutinate in response to ristocetin. No control or remission serum had this activity.

(*c*) Evidence for in vivo activity of CDP in TTP constituted part 3 of the study. Based upon the first two series of studies, we had evidence that TTP sera contained CDP and that serum CDP activity was highly sensitive and specific for TTP. However, the observation was an in vitro one. Because CDP was capable of cleaving platelet GPIb from platelets when studied in vitro, we measured this glycoprotein in two TTP patients. Platelet GPIb levels were reduced during the acute episode of TTP but returned to normal after recovery.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co, St Louis, except where stated otherwise. Platelet counts were measured on a Coulter S and platelet sizing was performed on a Coulter Channelizer (Coulter Electronics, Inc, Hialeah, FL). Fifteen patients with acute TTP were studied. All of these patients were tested at the initial presentation of their first episode of TTP before any plasma therapy had been given. Sera were available from five of these patients in remission of disease. Control sera for both the platelet release studies and ristocetin-induced agglutination inhibition studies were obtained from ten healthy volunteer donors, ten patients with idiopathic thrombocytopenic purpura, ten healthy females in

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the third trimester of pregnancy, four patients with preeclampsia, eight patients with disseminated intravascular coagulation, two patients with systemic lupus erythematosus and thrombocytopenia, ten patients with thrombocytopenia after cardiopulmonary bypass, two patients with heparin-induced thrombocytopenia, one patient with a hemolytic transfusion reaction (anti-E), two patients with extensive tissue damage due to severe trauma, and eight randomly selected nonthrombocytopenic hospital controls. In addition, normal RBC were suspended in a 2% suspension in autologous normal serum and lysed by sonication and the resultant solution tested in the release assay and in the ristocetin agglutination assay. For the ristocetin-induced agglutination inhibition studies, 392 additional randomly selected hospital patients were included as controls. Sera were separated into 1-mL aliquots and stored at −70°C. Aliquots were thawed at 37°C on the day of testing.

Preparation of control CDP from platelet lysates. Fifty units of platelet concentrates were pooled, washed, and disrupted by nitrogen cavitation.[4] CDP was purified as described.[5] The proteolytic activity of the CDP preparation was determined by using a ^{14}C-caseinolytic assay.[5] The purity of the preparation was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Studies of the effects of enzyme inhibitors on platelet ^{14}C-serotonin release. Normal pooled platelets were loaded with ^{14}C-serotonin (Amersham Corp, Oakville, Ontario)[6] with an average specific activity of 1.67×10^{-4} μCi/10^6 platelets (n = 3). The ability of test or control serum to cause release of ^{14}C-serotonin from labeled platelets was tested in a microtiter assay.[6] The following reagents were tested for their ability to inhibit the release caused by test sera: phenylmethylsulphonylfluoride (PMSF), 1 mmol/L; D-phenylanyl-1-prolyl-1-arginine chlormethyl ketone (PPAC) Calbiochem-Behring Corp, La Jolla, CA), 1 μmol/L; soybean trypsin inhibitor, 200 μg/mL; aprotinin, 100 Kallikrein-inhibition unit (KIU)/mL; iodoacetamide (IAA), 0.5 μmol/L; leupeptin (Calbiochem-Behring), 1 μmol/L antipain, 100 μmol/L; ϵ-aminocaproic acid (EACA), 0.2 μmol/L. All concentrations given are final concentrations. In each test the inhibitor and test serum were incubated together for ten minutes at 22°C before the addition of the ^{14}C-serotonin–labeled platelets. To rule out the possibility that the platelet release induced by TTP sera was due to platelet lysis, test platelets were labeled with ^{111}In as well as ^{14}C-serotinin and tested with serum from ten of the TTP patients. The platelets were labeled with ^{111}In by using the technique of Hawker et al[7] except that they were resuspended in phosphate-buffered saline (PBS) buffer and not plasma. The ^{111}In-labeled platelets had a specific activity of 4.3×10^{-5} mCi/10^6 platelets. The CDP preparation was tested at 70 and 1.5 caseinolytic U/mL for platelet lysis and release.

Confirmation of the presence of CDP activity in the serum of patients with TTP. The rationale of these experiments is that CDP can cleave glycocalicin from platelet GPIb, a change that would result in the platelets no longer being able to agglutinate in the presence of ristocetin and vWF. Formalin-fixed normal platelets were suspended in calcium- and albumin-free Tyrode's buffer (ph 7.45) to a final count of 300×10^9 platelets/L. The platelets were incubated with TTP or control sera that had been preincubated with buffer or with enzyme inhibitors. Test serum (0.25 mL) was preincubated with 75 μL buffer or inhibitor solutions for ten minutes at 22°C in the presence of 1 mmol/L $CaCl_2$. The serum was then added to 0.5 mL of platelet suspension in the presence of 1 mmol/L $CaCl_2$ The enzyme inhibitors tested in this series of experiments were the same as before except that EDTA (5 mmol/L) and *N*-ethylmaleamide (NEM) (6 mmol/L) were included. The serum/platelet mixture was incubated at 22°C for 60 minutes. The mixture was then centrifuged (2,100 g for 20 minutes), the supernatant discarded, and the platelets resuspended in calcium- and albumin-free Tyrode's buffer (pH 7.45, 3.00×10^9 platelets/L). These pretreated platelets were then tested for their ability to agglutinate in the presence of ristocetin and vWF[8] as follows: 0.1 mL of platelet suspension, 0.1 mL normal plasma, 30 μL ristocetin (H. Lundbeck A/S, Copenhagen) (1.5 mg/mL, final concentration) were mixed in a Payton aggregometer (Payton Associates, Scarborough, Ontario) at 37°C. After ten minutes, if no aggregation had taken place, 0.1 mL of nonpretreated fixed normal platelets was added to the aggregometer cuvette. Purified CDP (70 caseinolytic U/mL and 1.5 caseinolytic U/mL[5] and plasmin (5 U/mL) (Kabi, Stockholm) were also tested in place of test serum in these experiments.

Evidence for in vivo activity of CDP in TTP. Platelets from a patient with acute TTP before plasma therapy (platelet count, 20×10^9/L) were separated from blood drawn into acid-citrate-dextrose (ACD) with NEM (6 mmol/L) and leupeptin (1 μmol/L) and resuspended in calcium- and albumin-free Tyrode's buffer (pH 7.4) at a concentration of 120×10^9 platelets/L; these platelets were tested for their ability to agglutinate in the presence of ristocetin by using standard techniques. This assay was repeated when the patient entered remission.

The same platelets were also assayed for glycocalicin content by SDS-PAGE (4% stacking gel over a 5% to 15% gradient). A quantity of 6.4×10^8 platelets were labeled with ^{3}H and solubilized in 2% SDS with NEM (6 mmol/L). Control platelets from a healthy donor were treated identically. The accuracy of the number of platelets solubilized and applied to the gel was confirmed by platelet sizing and by light and phase microscopy to rule out red cell fragment contamination.

We also assayed the platelet membrane GPIb content in a second patient with TTP by radioimmune precipitation using the monoclonal anti-GPIb antibody 6D1 (generously provided by Dr B. Colleer, State University of New York at Stony Brook) as described.[9] Platelets from acute disease (platelet count, 20×10^9/L) and remission after plasmapheresis (platelet count, 257×10^9/L) were collected from blood drawn into inhibitors as before. The platelets were frozen in dimethylsulfoxide and autologous plasma and tested together on the same day.

RESULTS

Characterization of the platelet-activating factor by the effect of enzyme inhibitors on platelet ^{14}C-serotonin release. The sera from all 15 patients with TTP had platelet-activating activity as measured by the ^{14}C-serotonin release assay (Table 1). No platelet-releasing activity was detected in any of the control sera except from one of the two patients with heparin-induced thrombocytopenia and from the patient with anti-E. Sera from five of the TTP patients in remission (after plasma therapy had been discontinued) had no platelet-activating activity. A preparation of CDP at a concentration of 1.5 caseinolytic U/mL[5] also caused platelet aggregation and release; at a concentration of 70 caseinolytic U/mL the enzyme caused platelet lysis. Lysis (measured by ^{111}In release) did not occur at the lower concentration. SDS-PAGE analysis of the CDP preparation showed a major band at 76 kilodaltons (kD). Bands were also present at 118 kD and 64 kD, with some larger molecular weight proteins also present. The preparation was free of contaminating protease activity. The platelet release caused by the sera of patients with TTP during active disease and by the CDP preparation was consistently inhibited by leupeptin, IAA, and antipain but was not inhibited by PPAC, PMSF, aprotinin, soybean trypsin inhibitor, or EACA (Table 1). Three of these patients who entered remission later relapsed. Iden-

Table 1. Effects of Inhibitors on the Induction of ^{14}C-Serotonin Release From Normal Platelets by Sera From 15 Patients With TTP

Patients	PBS (pH 7.4)	PMSF (1 mmol/L)	PPAC (1 μmol/L)	Aprotinin (100 KIU/L)	EACA (0.2 μmol/L)	Soybean Tryspin Inhibitors (200 μg/ml)	IAA (0.5 μmol/L)	Leupeptin (1 μmol/L)	Antipain (100 μmol/L)
1	99	64	88	61	89	91	7	0	3
2	100	100	82	91	100	83	1	17	5
3	100	99	90	66	65	95	2	0	2
4	100	98	100	100	100	86	0	5	5
5	100	86	99	90	97	100	0	0	5
6	100	79	81	90	96	94	3	4	3
7	78	65	68	71	60	96	3	3	4
8	99	89	92	87	88	100	6	0	3
9	100	100	98	100	100	89	2	2	3
10	100	100	66	100	100	84	15	0	0
11	64	100	89	94	83	87	5	5	3
12	58	72	63	88	61	99	1	1	8
13	56	93	95	91	100	99	1	0	7
14	58	74	100	90	100	100	1	2	6
15	76	95	93	83	100	97	2	2	8

Concentrations are given as final concentrations. Inhibitors were incubated with test sera for ten minutes at 22°C before the addition of labeled platelets. Release for PBS is expressed as a percentage of the total possible release, ie, the amount of isotope taken up by the platelets. Release for the inhibitors is expressed as a percentage of the release in PBS. All tests were done in triplicate on two or more occasions. Serum from ten TTP patients (1, 2, 3, 4, 5, 7, 8, 10, 11, 13) were incubated with platelets labeled with ^{111}In as well as ^{14}C-serotonin to determine whether there was any platelet lysis occurring. These sera caused less than 1% release of ^{111}In.

tical platelet-releasing activity recurred. TTP sera did not cause ^{111}In release at the same time as ^{14}C-serotonin was released from test platelets, which indicated that platelet lysis was not taking place. The release induced by the sera from the patient with heparin-induced thrombocytopenia and from the patient with anti-E was inhibited by monomeric IgG (2 mg/mL) but was not affected by PMSF and leupeptin. IgG did not inhibit the TTP platelet release, as previously reported.[6]

Confirmation of the presence of CDP activity in the serum of patients with TTP. Formalin-fixed normal platelets preincubated with sera from all 15 TTP patients during periods of active disease and preincubated with purified CDP lost their ability to agglutinate in the presence of ristocetin and normal plasma (Fig 1). None of the control sera or the five TTP remission sera prevented the platelets from agglutinating. Nonpretreated formalin-fixed platelets added to the mixture of nonagglutinating pretreated platelets, normal plasma, and ristocetin in the aggregometer agglutinated normally, which indicated that the inhibition of agglutination was due to an effect on the preincubated platelets. Preincubation of TTP sera with EDTA, IAA, NEM, antipain, and leupeptin resulted in a loss of the inhibition of ristocetin-induced platelet agglutination in all 15 patients. Preincubation with PMSF, PPAC, aprotinin, EACA, or soybean trypsin inhibitor had no effect (Fig 1). Plasmin, 5 U/mL, also inhibited ristocetin-induced platelet agglutination; in contrast to TTP sera and CDP, plasmin was inhibited by PMSF, EACA, aprotinin, soybean trypsin inhibitor, antipain, and leupeptin but not by IAA or NEM. No change was seen in the platelet counts or in the distribution of the platelet size of the fixed normal platelets before and after exposure to TTP sera or CDP, which indicated that fragmentation of the platelets was not occurring.

Evidence for in vivo activity of CDP in TTP. SDS-PAGE analysis of the platelets from a patient with TTP is shown in Fig 2A. The patient's platelets show a marked loss of GP1b from the membrane. Other membrane proteins have

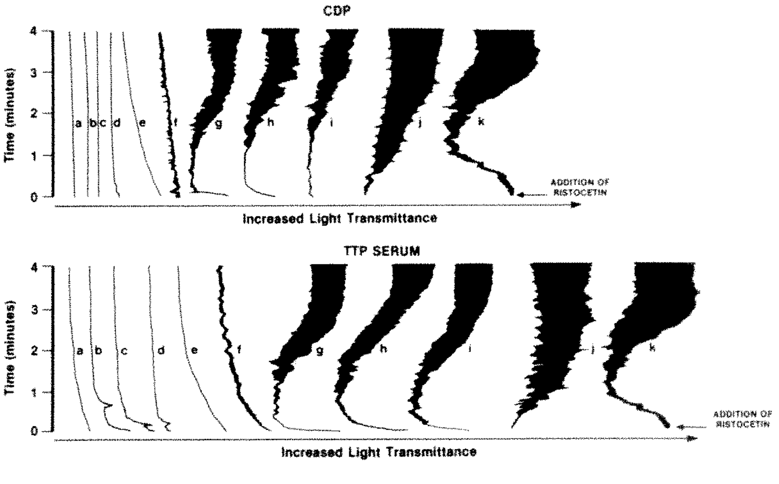

Fig 1. Effect of TTP serum and purified CDP with and without protease inhibitors on ristocetin-induced platelet agglutination. Protease inhibitors are (a) buffer, (b) PMSF, (c) PPAC, (d) EACA, (e) soybean trypsin inhibitor, (f) aprotinin, (g) IAA, (h) leupeptin, (i) NEM, (j) EDTA, and (k) a buffer control without added serum. Test sera, with and without inhibitors, were incubated with formalin-fixed normal platelets at 22°C for 60 minutes. The treated platelets were centrifuged and then resuspended in calcium- and albumin-free Tyrode's buffer (pH 7.45) at a count of 300 × 10^9 platelets/L; agglutination of the platelets in the presence of ristocetin and normal plasma was recorded in an aggregometer.

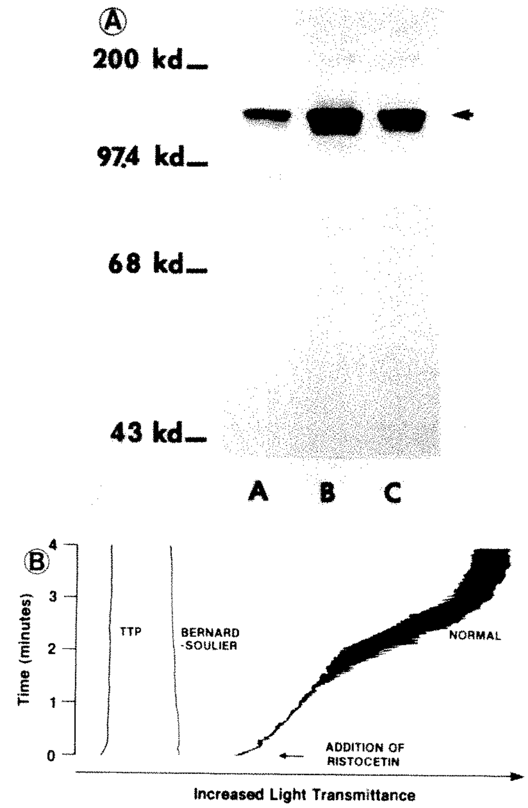

Fig 2. (A) SDS-PAGE of platelet membrane glycoproteins from a patient with TTP before treatment. Quantities of 6.4 × 10^8 platelets from the patient (lane A) and from a normal control (lane B) were labeled with ^3H and solubilized in 2% SDS. Lane C is an added control: 3.2 × 10^8 platelets from the same normal control were applied to this lane after labeling and solubilization. There is a marked loss of GP1b from the patients platelets (arrow). The electrophoretic mobility of this glycoprotein under reduced and nonreduced conditions was characteristic of GP1b.[10] Other labeled membrane proteins have also been decreased. An additional unidentified protein band is seen in the patient sample at 90 kD. (B) Ristocetin-induced agglutination of the patient's platelets. The platelets were suspended in calcium- and albumin-free Tyrode's buffer at a concentration of 120 × 10^9/L and tested for agglutination in the presence of normal plasma and ristocetin (1.5 mg/mL). Control normal platelets and platelets from a patient with Bernard-Soulier syndrome (lacking GP1b) were collected and tested identically. Blood for these analyses was drawn into ACD with leupeptin (1 μmol/L) and NEM (6 mmol/L).

also been lost from the platelets. The patient's platelets did not agglutinate in the presence of ristocetin (Fig 2B). After remission, the patient's platelets agglutinated normally.

Radioimmune precipitation analysis of platelets from a second patient studied during active disease and in remission also showed a decrease of detectable GP1b on the platelets during active disease and a return to normal levels during remission (data not shown).

DISCUSSION

TTP is characterized by disseminated platelet thrombi within the circulation; it is possible that a platelet-activating factor could participate in the pathogenesis of this disease. The studies described in this report suggest that this factor is CDP. We have demonstrated the presence of CDP in the sera of patients with TTP in several ways. First, we studied the effect of enzyme inhibitors on the platelet release reaction induced by TTP sera. Cysteine protease inhibitors but not serine protease inhibitors consistently inhibited platelet release. We then confirmed that CDP activity was present in TTP sera by demonstrating that TTP sera possessed the ability to proteolyse GP1b and that this proteolytic activity was calcium dependent and was sensitive to inhibition by cysteine protease inhibitors but not by serine protease inhibitors. The CDP activity was highly specific to TTP: we detected no activity in 459 control sera, including 36 patients with thrombocytopenia of various causes and more than 400 randomly selected hospital patients. Further, no activity was present in the sera of five of the TTP patients in remission, although it recurred in three patients upon relapse.

Platelets from two patients with TTP (from blood drawn directly into CDP inhibitors) showed a loss of GP1b from the platelet membrane during active disease. Platelets (tested in one patient) also showed a loss of the ability to agglutinate in the presence of ristocetin, comparable to that seen in Bernard-Soulier platelets. These observations are consistent with the enzyme-induced loss of glycocalicin from the platelet membrane and suggest that CDP is active in vivo in patients with TTP.

CDP is a cytosolic enzyme found normally in several tissues including platelets.[11] Its exact physiological role is uncertain, though it is known to be capable of proteolysis of a number of proteins including platelet membrane glycoproteins.[4] It has been demonstrated previously[4] and we have confirmed that the enzyme has platelet-activating activity. The pathway of aggregation is via platelet GPIIb/IIIa.[4] This is consistent with our previous observations[6] that platelet activation induced by TTP serum is inhibited by a monoclonal antibody against GPIIb/IIIa but not by anti-GP1b monoclonal antibody. It is likely, therefore, that platelet aggregation is the major in vivo effect of CDP on the platelet in TTP and that the effect on GP1b is a secondary one.

It is uncertain how our current studies showing CDP activity in TTP relate to previous observations. Several investigators have implicated vWF as an active or passive participant in TTP. Abnormally large multimers of vWF are present in the circulation of convalescent patients with recurrent TTP.[2,12,13] Because the large multimers disappear during acute episodes of the illness, it has been postulated that vWF directly participates in the intravascular platelet clumping that characterizes TTP. In addition, we have shown that the platelet-aggregating activity of TTP can be enhanced in vitro by the addition of a preparation rich in large multimers of vWF.[2] And yet, CDP has been demonstrated to cleave vWF, thereby resulting in the loss of the largest multimers.[14] Therefore, it is possible that the abnormalities of vWF in TTP patients represent an epiphenomenon and are a consequence of CDP activity. However, these issues must await further investigations for their resolution.

On the basis of the findings described in these studies, we postulate that platelet aggregation in TTP is generated through a disturbance of CDP homeostasis that leads to the presence of enzyme activity in the serum. Our studies also indicate that inhibition of ristocetin-induced platelet agglutination is a sensitive and specific assay for TTP.

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CONCISE REPORT

A Novel Globin Structural Mutant, Showa-Yakushiji ($_\beta$110 Leu-Pro) Causing a β-Thalassemia Phenotype

By Yasushi Kobayashi, Yasuyuki Fukumaki, Norio Komatsu, Yuzo Ohba, Takaoki Miyaji, and Yasusada Miura

Molecular analysis of the β-globin genes from a patient with a β-thalassemia phenotype showed that a single nucleotide mutation (CTG-CCG) at codon 110 in one of the genes resulted in a leucine to proline substitution. The same mutation with a similar phenotype, was observed in her mother and sister, by Southern blotting analysis of DNAs digested with *MspI*, the recognition site of which was created by this base substitution. This indicates a close relationship between this mutation and the β-thalassemia phenotype. No anomalous peak of radioactivity was detected by reverse-phase high-performance liquid chromatography (HPLC) in the patient's reticulocytes incubated with isotopically labeled amino acid. The leucine-proline (Leu-Pro) substitution probably disrupts the G-helix and in turn interferes with globin contact points. The uncombined β-globin chain would be rapidly destroyed and the β-thalassemia phenotype would follow.

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THALASSEMIA MUTATIONS are traditionally defined as those that leave the globin structure unchanged, but lead to a quantitative reduction in the synthesis of globin. In contrast, hemoglobinopathies are defined as abnormal globins with one or more structural changes, which are synthesized at a normal rate. This classification of globin gene mutations is not always apt as there are several mutations that cause not only alterations of the globin structure but also a decrease in synthesis. These are known as thalassemic hemoglobinopathies and the molecular mechanisms are activation of the splice site within the exon, loss of a normal termination or instability of the globin chains.[1] The highly unstable globin mutants causing a thalassemic phenotype are of great interest as the posttranslational defect occurs at the most distal step necessary for the functional expression of the globin genes and vital information on the effect of the amino acid substitution on the hemoglobin tetramer formation can be obtained. We report herein a β-globin gene with a novel mutation, 110 leucine-proline (Leu-Pro) that causes a β-thalassemia phenotype, through a posttranslational mechanism.

MATERIALS AND METHODS

Hematologic and hemoglobin procedures. Standard hematologic measurements were performed. Hemoglobin electrophoresis was carried out on a starch gel at pH 8.6[2] and polyacrylamide gel isoelectric focusing.[3] HbF and HbA_2 levels were determined as described.[4] Stability of hemoglobin was examined by heat denaturation[5] and by the isopropanol test.[6] Glycerol lysis time (GLT_{50} value) of erythrocytes was determined as described.[7] Incubation of blood samples with L-[4.5-^{3}H] leucine (40 to 50 Ci/mmol) for two hours and each labeled globin chain was separated by CM-cellulose chromatography eluted with 8 mol/L urea buffer to determine the globin chain synthesis ratio.[8]

DNA analysis. High-molecular-weight DNA was extracted from the peripheral leukocytes of the proband, family members, and individuals with no hematologic abnormalities, as described.[9] The β-globin genes of the proband were cloned in bacteriophage Charon 28 as 7.8 kb *HindIII* fragments. Nucleotide sequence of the β-globin genes was determined by the dideoxy chain termination method.[10] Restriction site polymorphisms within the β-globin gene cluster and creation of the new restriction site in the β-globin gene by one base substitution were determined by Southern blotting procedures, using appropriate probes.[11]

Globin chain analysis by reverse-phase HPLC. Globin chains were separated from the fresh hemolysate by reverse-phase high performance liquid chromatography (HPLC) on a TSK gel ODS-120T column in 0.1% trifluoroacetic acid (TFA) with an acetonitrile gradient of 42.2% to 48.2% in 60 minutes.[12,13] The β-globin chain prepared by a CM-cellulose column was S-alkylated with iodoacetamide and then digested with trypsin.[14] The resultant digests were also isolated by reverse-phase HPLC on a μBondapak C18 in 0.05% TFA with an acetonitrile gradient of 0% to 48% in 180 minutes.[12]

RESULTS

Hematologic data of patients. The proband is a 34-year-old Japanese woman who was admitted to the hospital because of anemia. The hematologic findings and other laboratory data of the proband, her sister, her mother, and her father are presented in Table 1. These data of the proband resembled those associated with β-thalassemia trait and were inconsistent with the presence of a significant hemolytic process due to an unstable hemoglobin. Similar hematologic findings were observed in her mother and sister but not in her father, suggesting that the mother and sister were heterozygous for the same mutation.

Structural analysis of the β-globin genes. To define the molecular lesion producing a β-thalassemia phenotype, we isolated several β-globin gene clones of the proband and chose two (clone A and clone B) that were distinguished by the polymorphism of the *AvaII* site located in position 16 of IVS2. We determined the nucleotide sequence that extends

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Table 1. Hematologic and Other Laboratory Data of the Proband and Her Family Members

	Proband	Sister	Mother	Father
Hb (g/dL)	7.6	9.8	9.0	14.4
RBC (× 10^{12}/L)	4.29	5.12	4.50	4.67
MCV (fL)	56.0	60.0	60.0	90.0
MCH (pg)	17.8	19.1	20.0	30.8
MCHC (g/dL)	31.3	31.5	33.2	34.4
Reticulocyte (%)	2.4	4.1	2.1	0.7
Birilubin (mg/dL)	0.46	0.72	0.50	0.55
LDH (IU/L)	276	261	285	324
HbF (%)	1.3	2.0	2.0	ND
HbA$_2$ (%)	5.2	5.3	5.3	ND
Heat denaturation test (50°C) (%)	(−)	(−)	(−)	ND
Isopropanol test	(−)	(−)	(−)	ND
Electrophoresis (starch, isoelectric)	No abnormal bands			ND
GLT$_{50}$ value (s)	109	99	79	ND
β/α chain synthesis ratio	0.8	0.8	0.7	ND

Abbreviations: MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; GLT, glycerol lysis time; ND, not determined.

from 372 bases 5′ of the cap site to 266 bases 3′ of the polyA site of both clones. Comparison of the nucleotide sequence with that of several human β-globin genes[15,16] revealed one notable nucleotide substitution (T-C) at the second position in codon 110 of clone A (Fig 1). Orkin *et al* reported that the β-globin gene exhibits polymorphism limited almost exclusively to common sequence types, which they designated as β-globin gene frameworks.[17] According to their definition, clone A sequence and clone B sequence were framework 3 Asian and framework 1, respectively. We analyzed the polymorphic restriction sites in the β-globin gene cluster (the *Hinc*II site 5′ to the ε-globin gene, the *Hind*III sites in the G_γ−, A_γ− globin genes, the *Hinc*II site in the ψβ-globin gene, the *Hinc*II site 3′ to the ψβ-globin gene, the *Hgi*AI, *Ava*II sites in the β-globin gene and the *Bam*HI site 3′ to the β-globin gene) to determine the haplotype of the chromosome bearing the clone A sequence. We found that the haplotype (+ − − − − − − +) was linked to this mutation allele.

Association of the mutation with the β-thalassemia phenotype. The base change at codon 110 converts the DNA sequence in the region from CTGG to CCGG, thereby creating the *Msp*I recognition site. Hence, digesting clone A DNA with both *Msp*I and *Hind*III produces a 3.9 kb fragment instead of a 4.7 kb fragment (Fig 2). To examine the association of this mutation with the β-thalassemia phenotype, we digested the genomic DNAs of family members with both *MspI* and *Hind*III and performed Southern blotting analysis of each digested DNA, using a 5′-specific β-globin gene probe (a 1.9 kb *Bam*HI fragment). This *Msp*I assay demonstrated that the proband's mother and sister but not her father were also heterozygous for this mutation (Fig 2). These results indicate a close relationship between the mutation and the thalassemia phenotype.

Analysis of globin chains using reverse-phase HPLC. To investigate features of the mutant globin chain predicted from the nucleotide sequence, peripheral blood of the proband was incubated with L-^{3}H-leucine for 15 minutes, and 120 minutes at 37°C. The labeled globin chains were analyzed with reverse-phase HPLC, under conditions that separate the unstable β-globin chain of Hb Santa Ana (β88

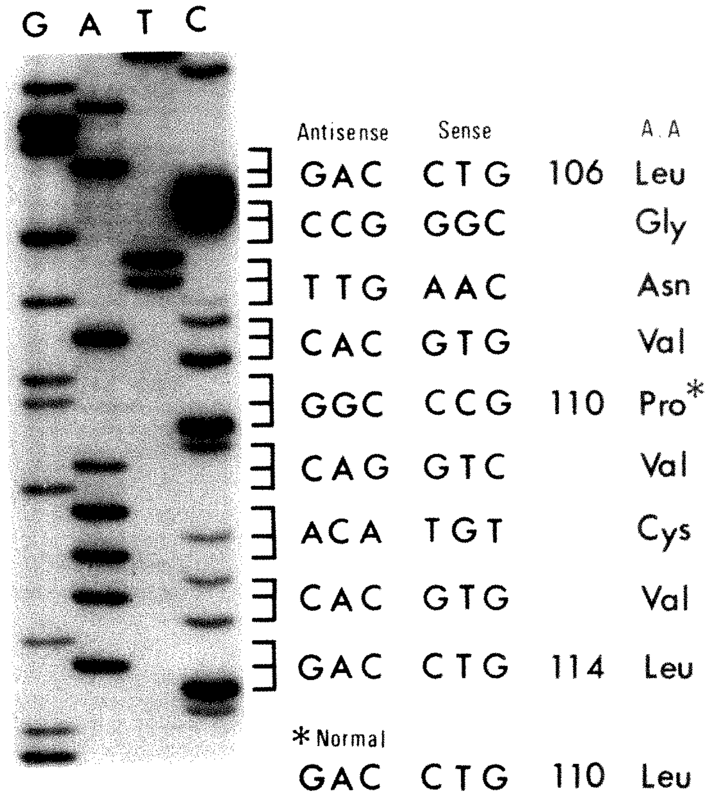

Fig 1. DNA sequence of the β-globin gene (clone A) of the proband and the autoradiogram of the gel demonstrating the sequence of the antisense strand in the vicinity of the codon 110. The T-C substitution at the second position of condon 110, that results in the substitution of proline for leucine.

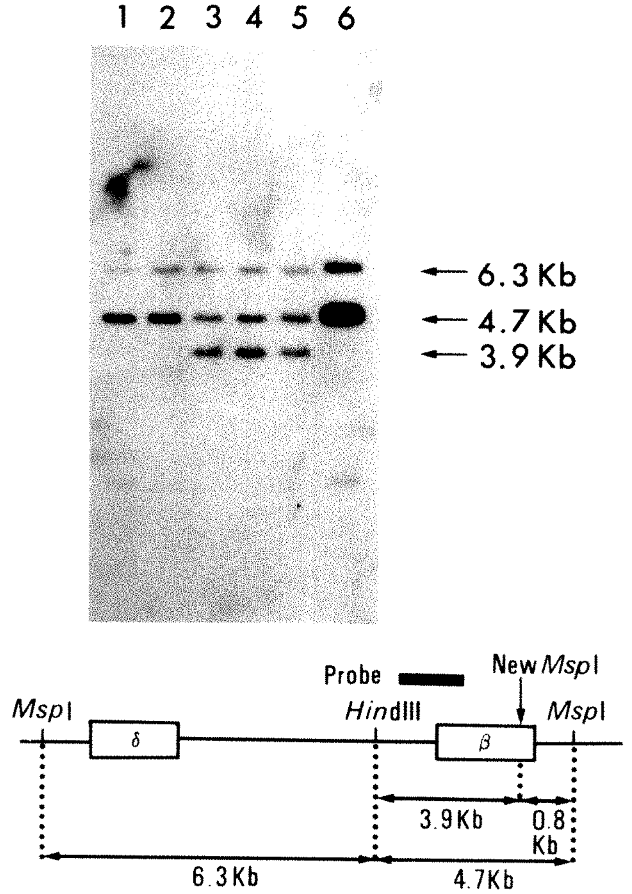

Fig 2. Identification of the mutation at codon 110 by Southern blotting. At the top is an autoradiogram of DNA fragments that hybridize with a 5′-specific β-globin gene probe after *Msp*I and *Hind*III digestion of DNAs of the proband (lane 5), her sister (lane 4), her mother (lane 3), her father (lane 2), and normal controls (lanes 1 and 6). The 4.7 kb fragment was seen in normal DNAs and the 3.9 kb fragment in patients' DNAs. The 6.3 kb band is due to cross-hybridization with the δ-globin gene sequence. At the bottom is a map of *Msp*I and *Hind*III sites in the region containing the δ- and β-globin genes. The position of the *Msp*I site created by the base substitution at codon 110 is indicated by the arrow with new *Msp*I.

Leu-Pro)[18] from the normal counterpart. We also analyzed globin chain labeled with L-^{3}H-proline, under the same conditions. Chromatographic analysis revealed no anomalous peak of radioactivity in the reticulocytes incubated for 15 minutes (data not shown) and 120 minutes (Fig 3). To confirm these results, we analyzed tryptic peptides of the β-globin chain labeled with L-^{3}H-leucine and L-^{3}H-proline, separately, by reverse-phase HPLC, as described.[12] The radioactivity was consistent with the tryptic peptides that contain leucine at amino acid 110 and there was no abnormal hyperactive peak (data not shown). These results rule out the possibility that the mutant globin comigrated electrophoretically with the normal β-globin because of the lack of charge involved in the Leu-Pro substitution. The possibility that the mutant globin chain is extremely labile is given support.

DISCUSSION

A single nucleotide change found at codon 110 causes the leucine to proline substitution in individuals with a β-thalassemia phenotype. No abnormal globin chain was detected in reticulocytes even after 15 minutes labeling with L-^{3}H-leucine, by reverse-phase HPLC under conditions that separated the Leu-Pro variant from the normal globin chain. Proline (Leu-Pro) substitutions usually exert a profound effect on stability of the hemoglobin tetramer, because proline residues are unable to form an α-helix and are at the inter-helical segments of the chain where the helix has to be broken.[19] The mutation studied here is the thirteenth reported example of a Leu-Pro substitution in globin chains.[20] All the known Hb mutants with this amino acid substitution in the α- and β-globin chains produce unstable hemoglobin and cause hemolytic anemia,[20] except for the Hb Quong Sze.[21] The unusual feature of the mutation studied here is that the Leu-Pro substitution produces β-thalassemia rather than hemolytic anemia. Four α1β1 contact points, condons 108, 112, 115, and 116, are located in the G-helix of the β-globin chain.[22] These contact points are essential for initial dimeric formation and subsequently for the hemoglobin tetramer assembly. The substitution of proline for leucine at codon 110 probably disrupts the G-helix and in turn interferes with these contact points. The uncombined β-globin chain would be rapidly catabolized by proteolysis and the β-thalassemia phenotype would appear. A similar mechanism was described for the Hb Quong Sze (α125 Leu-Pro), which produces α-thalassemia rather than hemolytic anemia.[21] However, the proline substitutions at helical position G 8 (Hb Southampton-Casper),[23] G17 (Hb Madrid),[24] and G 19 (Hb Saitama)[14] produce hemoglobin variants present at levels of 20% to 40% and do not produce the thalassemic phenotype. The phenotypic difference between these variants and the mutation studied here could be derived from the position of the amino acid substitution in the G-helix. In our patient, the substitution of proline for leucine is located at G 12, almost at the center of the G-helix, so that a profound effect on the helical formation would be expected.

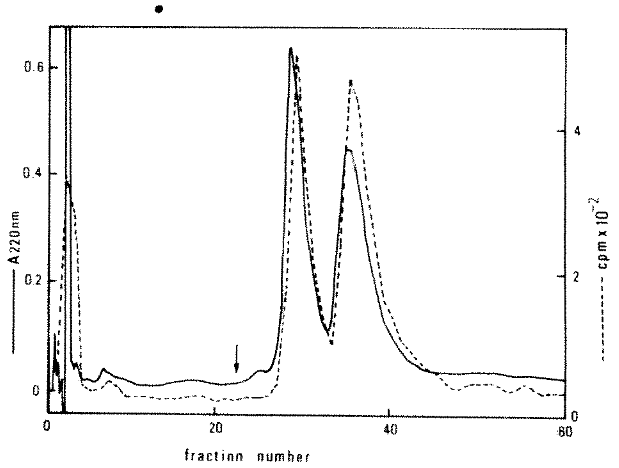

Fig 3. Chromatogram of the labeled globin chains. The peripheral blood of the proband was incubated in the presence of L-^{3}H-leucine (250 μCi/mL) for two hours at 37°C. The labeled globin chains were separated by reverse-phase HPLC on a TSK gel ODS-120T column. (——) Absorbance; (----) radioactivity. Arrow indicates the position of the abnormal β-globin chain (β88 Leu-Pro) of Hb Sant Ana.

A highly unstable β-globin mutant codon 112 (Cys-Arg), Hb Indianapolis, is also known to be associated with a β-thalassemia phenotype.[8] Heterozygotes for Hb Indianapolis have β/α-globin synthesis ratios of ~0.4. In contrast the globin chain synthesis ratios were higher than 0.7 in the cases we studied. These observations are not fully explained by the results of HPLC analysis. A condition that represses the α-globin chain production, such as in α-thalassemia, might be associated, although Southern blot analysis showed no abnormalities in the α-globin gene cluster of the proband (data not shown).

During the preparation of the manuscript, we learned from Dr Y. Naritomi et al that the same mutation has been found in an unrelated Japanese individual with a δβ-thalassemia phenotype. Therefore, we named this mutant, Showa-Yakushiji after names of the towns inhabited by each proband.

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CONCISE REPORT

Mixed Blood Chimerism in T Cell-Depleted Bone Marrow Transplant Recipients: Evaluation Using DNA Polymorphisms

By S. Bretagne, M. Vidaud, M. Kuentz, C. Cordonnier, T. Henni, G. Vinci, M. Goossens, and J.P. Vernant

We have used DNA sequence polymorphism analysis to document engraftment after T cell-depleted bone marrow transplantation (BMT), with a selected panel of four DNA probes. In contrast to nondepleted BMT recipients, the patients who received T cell-depleted marrow exhibited a mixed blood chimerism. This mosaicism was observed before graft failure or relapse in six patients. However, in five other patients, this mixed chimerism was not followed by these complications with a follow-up of 9 to 31 months after transplantation. Our results support the hypothesis that transplanted bone marrow T cells may help to maintain engraftment by eliminating host cells that can cause graft failure.

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THE ABILITY to establish the origin of cells following bone marrow transplantation (BMT) and to detect change in a patient's hematopoietic and lymphoid cell compartments from host to donor origin is clinically useful in assessing both the occurrence and completeness of engraftment. In particular, the pattern of engraftment is of interest with regard to the development of in vitro T cell depletion of the BMT. This technique, which has proved useful in the prevention of graft-*v*-host disease (GVHD),[1] dramatically increases the risk of graft failure and relapse,[2,3] a risk that is probably associated with the existence of residual host cells multiplying after transplantation. In order to explore this phenomenon, we have documented and characterized the pattern of engraftment in T cell-depleted and nondepleted BMT recipients using DNA sequence polymorphism analysis.

PATIENTS AND METHODS

Patients

Beginning in September 1984, certain patients transplanted in our unit received a T cell-depleted graft (Table 1). In this prospective study, recipients >30 years were selected for in vitro T cell depletion: 19 of these patients were analyzed for peripheral blood chimerism (12 in the depleted group and seven in the nondepleted group). In addition, three patients (patients no. 13, 14, and 15) having received a nondepleted transplant before the beginning of this study were examined retrospectively and are included in the control group.

All 22 patients received an allogeneic BMT from a HLA-identical mixed lymphocyte culture negative sibling as therapy for hematological malignant diseases (acute myeloblastic leukemia [AML], 7; acute lymphoblastic leukemia [ALL], 6; chronic myeloid leukemia [CML], 6; preleukemic state, 3). The BMT conditioning regimen consisted of chemotherapy (cyclophosphamide, 60 mg/kg × 2) and total body irradiation (TBI) (10 Gy single dose with mean dose rate 3 cGy/mn and lung shilding above 8 Gy). Six patients (no. 2, 5, 6, 10, 11, and 21) received a more ablative preparation (additive drugs or total lymphoid irradiation) (Table 1). All were given methotrexate (15 mg/m^2 on day 1, 10 mg/m^2 on days 3, 6, and 11, and weekly up to day 100) as GVHD prophylaxis. Twelve patients (no. 1 to 12) received T cell-depleted BMT either with monoclonal antibodies CD2-CD5-CD7 plus rabbit complement (patients no. 1, 2, 6, and 7) or with T101 immunotoxin (patients no. 3, 4, 5, 8, 9, 10, 11, and 12). These two techniques are equally efficient (mean depletion 99% and mean injected T cells 0.12 × 10^6/kg).[4] All patients were advised of chemotherapeutic and bone marrow procedures and their attendant risks, and gave informed consent.

DNA Analysis

In all the patients studied, engraftment was established and monitored with the use of DNA sequence polymorphism analysis as previously described[5-7] or with a Y chromosome-specific probe when donor and recipient sex were different. The genotype of the posttransplant cell fractions was compared with the pretransplant host and donor genotype.

Preparation of genomic DNA samples. Peripheral blood specimens from donors and patients before and after BMT were collected in EDTA. High molecular weight DNA was prepared by standard techniques. Ten micrograms of DNA were digested to completion with the restriction enzyme *Taq*I and 5 μg with *Hae*III in the case of sex mismatch. DNA fragments were then fractionated by electrophoresis in 1% agarose gels and transferred to nitrocellulose filters by the Southern's technique.

Hybridization with DNA probes. A panel of four probes comprising two X chromosome-specific probes (St14 and L1.28), a Y chromosome-specific sequence (HS5), and an autosomal probe (MetH) were used. The St14 probe for locus DXS52 (Xq28) is a 3.0 kg *Eco*R1 fragment that detects a multiallelic *Taq*I polymorphism.[8] Probe L1.28 detects a two alleles *Taq*I polymorphism at the locus DXS7 on the short arm of chromosome X.[9] The HS5 probe is a cloned DNA fragment that represents a major component of the human Y chromosome-specific repeated DNA family (DY21). Male DNA digested by Hae III contains 800 to 5,000 copies of a 3.4 kb DNA fragment,[10] a fragment that we never observed, in our experimental conditions, in DNA of female origin. The MetH probe detects a two allele *Taq*I polymorphism at a locus localized on the long arm of chromosome 7 (7q22-31).[11] The probes were labeled with ^{32}P deoxynucleotide triphosphates (Amersham International plc, Buckinghamshire, UK). Nitrocellulose filters were hybridized, washed, examined by autoradiography and the films were scanned with a spectrophotometer (Cellosystem, SEBIA, Issy-les-Moulineaux, France).

Fractionation of posttransplantation cells. In five patients (no. 3, 4, 8, 11, and 13) blood cells were fractionated into granulocytes, T

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Table 1. Patients Examined Post-BMT by DNA Analysis

Patient No.	Age/Sex	Diagnosis	Status at BMT	Donor Age/Sex	Preparation CTX/TBI	T Cell Depletion	GVHD Acute/Chronic	Date of Study Post-BMT	DNA Probes	Chimerism	Follow-up
1	40/F	ALL	1st R	39/F	+	+	0/0	17 mo	S	M	>31 mo
2	44/F	CML	1st CP	46/M	+ TLI	+	0/0	7, 10, 12, 14 mo	S	M	>17 mo
3	40/M	ALL	1st R	36/F	+	+	0/0	9, 12 mo	H	M	>15 mo
4	34/M	CML	1st CP	51/F	+	+	0/0	1, 3, 8 mo	H	M	>12 mo
5	34/M	CML	1st CP	32/F	+ CCNU/AraC	+	2/0	1, 3 mo	HS	M	>9 mo
6	40/F	AML	2nd R	26/M	+ VP16/CCNU	+	0/0	4 mo	S	M	Relapse at 5 mo
7	36/F	ALL	1st R	29/M	+	+	0/0	4 mo	S	M	Relapse at 5 mo
8	31/F	AML	2nd R	38/M	+	+	0/0	2, 3, 5 mo	S	M	Relapse at 4 mo
9	35/M	CML	1st CP	32/F	+	+	0/0	3, 4 mo	HS	M	Graft failure at 4 mo
10	35/F	Preleukemic state		33/M	+ CCNU/AraC	+	0/0	60, 75, 103, d	SH	M	Graft failure at 2 mo
11	39/F	Preleukemic state		37/M	+ TLI	+	0/0	3, 4, 8 mo	SH	M	Graft failure at 3 mo
12	18/M	CML	1st CP	15/M	+	+	0/0	1, 2 mo	SLM	NE	8 mo
13	15/M	ALL	1st R	17/F	+	–	0/0	60, 72 mo	H	M	>72 mo
14	13/M	AML	1st R	14/F	+	–	1/0	30 mo	H	T	>36 mo
15	27/M	CML	1st CP	33/F	+	–	4/2	32 mo	H	T	36 mo
16	20/M	AML	1st R	19/F	+	–	1/0	3, 15 mo	H	T	>15 mo
17	17/M	ALL	2nd R	20/M	+	–	1/0	76 d	L	T	>9 mo
18	37/M	ALL	1st R	38/M	+	–	3/0	63 d	L	T	Deceased on 70 d
19	19/F	AML	1st R	19/M	+	–	2/0	30 d	S	T	>8 mo
20	50/F	Preleukemic state		37/F	+	–	1/0	56 d	S	T	Relapse at 6 mo
21	41/F	AML	1st R	39/M	CTX/Misulban	–	2/0	49 d	S	T	>7 mo
22	31/M	AML	2nd R	38/M	+	–	2/0	1, 2 mo	SLM	NE	>9 mo

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CML, chronic myeloid leukemia; R, remission; CP, chronic phase; CTX, Cyclophosphamide (60 mg/kg, days −5 and −4); Ara,C, cytosine arabinoside (200 mg/m^2, days −5, −4, −3, and −2); CCNU, lomustine (150 mg/m^2, day −5); VP16, etoposide (200 mg/m^2, days −5, −4, and −3); TBI, total body irradiation (10 Gy single dose, day −1); TLI, total lymphoid irradiation (6 Gy in four fractions, days −10, −9, −8, and −7); M, mixed; T, total; NE, not evaluable; S, St14 (DXS52); L, L1.28 (DXS7); H, HS5 (DYZ1); M, Met H.

and B lymphocytes by Ficoll centrifugation, E-rosetting, and plastic adherence.

RESULTS

We first estimated the sensitivity of the assay by dilution experiments in which patient and donor DNAs were mixed in varying proportions. The minor cell population is still visible at levels in the order of 1% to 5% of total DNA with the use of the probes St14, L1.28, and MetH. This level of sensitivity is equivalent to that described by others using similar techniques.[5-7] In contrast, the use of probe HS5, in our experimental conditions, allows detection of 1 to 10 ng of male DNA. We evaluated that the level of sensitivity attained is as high as 0.1%, ie, a hybridization fragment was detectable in at least 0.1% of the cells from which DNA was extracted. Thus, this probe is very useful in the detection of residual male cells (residual cells of a male recipient transplanted from a female donor or cells of a male BMT in a female recipient).

The chimerism was analyzed with the HS5 probe in eight male recipients of female donor (patients no. 3, 4, 5, 9, 13, 14, 15, and 16), with St14 in ten female recipients of either male (patients no. 1 and 20) or female donor (patients no. 2, 6, 7, 8, 10, 11, 19, and 21) and with L1.28 in two male recipients of male donor (patients no. 17 and 18). In the two remaining male/male pairs (patients no. 12 and 22) these probes and MetH failed to distinguish between recipient and donor DNA.

In four recipients, the use of both St14 and HS5 probes was necessary to quantify the chimerism. In patient no. 9, male host cells exceeded 10% of total cells as demonstrated by the intensity of the hybridization signal with the Y-specific sequence, and probe St14 was used to evaluate the proportion of female donor DNA. In patients no. 10 and 11, probe St14 did not detect any DNA of male origin but the use of probe HS5 showed that engraftment of male cells persisted although clinical diagnosis of graft rejection had been made (up to 5 months after clinical diagnosis of graft rejection for patient no. 11). In patient no. 5, probe St14, in contrast to probe HS5, failed to detect residual male host cells at day 30 but could detect a male-specific allele at day 90 (Fig 1). It thus appears that the precocity with which the diagnosis of partial chimerism can be made depends on the sensitivity of the test used and on the growth rate of the residual cell population. Of the nine nondepleted BMT recipients studied (patients no. 13 to 21), eight exhibited total blood chimerism (from 2 months to 3 years post-BMT) and one (patient no. 13) (ALL) displayed a partial chimer-

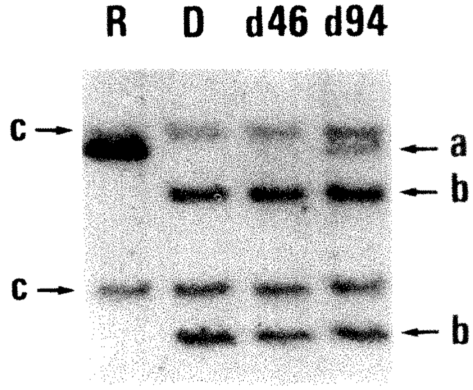

Fig 1. Expansion of residual host cells after transplantation (patient no. 5). DNA samples were obtained from the female donor (D) and from the male recipient prior to BMT (R) and after transplantation on day 46 (d46) and on day 94 (d94). Following digestion with Taq1 and hybridization with the probe St14 (DXS52) the recipient-specific allele (a) absent on day 46, was detected on day 94 post BMT (b_1 and b_2: donor specific alleles; c: constant fragments).

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In four recipients, the use of both St14 and HS5 probes was necessary to quantify the chimerism. In patient no. 9, male host cells exceeded 10% of total cells as demonstrated by the intensity of the hybridization signal with the Y-specific sequence, and probe St14 was used to evaluate the proportion of female donor DNA. In patients no. 10 and 11, probe St14 did not detect any DNA of male origin but the use of probe HS5 showed that engraftment of male cells persisted although clinical diagnosis of graft rejection had been made (up to 5 months after clinical diagnosis of graft rejection for patient no. 11). In patient no. 5, probe St14, in contrast to probe HS5, failed to detect residual male host cells at day 30 but could detect a male-specific allele at day 90 (Fig 1). It thus appears that the precocity with which the diagnosis of partial chimerism can be made depends on the sensitivity of the test used and on the growth rate of the residual cell population. Of the nine nondepleted BMT recipients studied (patients no. 13 to 21), eight exhibited total blood chimerism (from 2 months to 3 years post-BMT) and one (patient no. 13) (ALL) displayed a partial chimer-

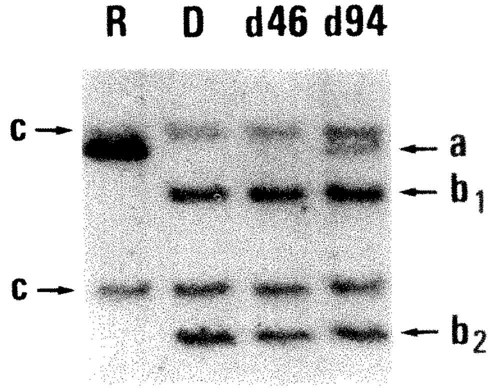

Fig 1. Expansion of residual host cells after transplantation (patient no. 5). DNA samples were obtained from the female donor (D) and from the male recipient prior to BMT (R) and after transplantation on day 46 (d46) and on day 94 (d94). Following digestion with Taq1 and hybridization with the probe St14 (DXS52) the recipient-specific allele (a) absent on day 46, was detected on day 94 post BMT (b_1 and b_2: donor specific alleles; c: constant fragments).

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CONCISE REPORT

The Effect of Recombinant GM-CSF on the Recovery of Monkeys Transplanted With Autologous Bone Marrow

By R.L. Monroy, R.R. Skelly, T.J. MacVittie, T.A. Davis, J.J. Sauber, S.C. Clark, and R.E. Donahue

The regulatory function of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) on granulocyte production in vivo was evaluated in an autologous bone marrow transplantation model using rhesus monkeys. Monkeys were exposed to 9.0 Gy total body irradiation and then transplanted with 5.0 × 10^7 low-density bone marrow cells/kg. Alzet miniosmotic pumps were subcutaneously implanted to deliver rhGM-CSF at a rate of 50,400 U/kg/d. Minipumps, containing either rhGM-CSF or saline, were implanted between zero and five days after transplantation for seven days. Kinetic recoveries of peripheral blood cells after either saline or rhGM-CSF treatment were compared. Treatment with rhGM-CSF accelerated the recovery of neutrophils. Neutrophils in rhGM-CSF-treated animals recovered to 80% (3.4 × 10^3/mm^3) pre-irradiation control levels by day 20, in comparison with only 33% (0.9 × 10^3/mm^3) recovery for saline control monkeys. In addition, the recovery of neutrophils was enhanced over that of the controls, reaching 140% v 70% on day 30. Another prominent feature of rhGM-CSF-treated monkeys was the accelerated recovery of platelets, reaching near 50% normal levels by day 24 in comparison with 20% of normal levels for controls. The infusion of rhGM-CSF was shown to be an effective regulator of early hematopoietic regeneration, leading to the accelerated recovery of both neutrophils and platelets and then providing a consistent sustained increase of neutrophils even in the absence of rhGM-CSF.

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A VARIETY of glycoproteins with hormone-like stimulatory effects on hematopoietic progenitor cells have been purified and characterized. In recent articles, the molecular and biochemical properties of a specific murine and human glycoprotein, granulocyte-macrophage colony stimulating factor, were comprehensively reviewed.[1-3] Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) has been shown to stimulate the proliferation of granulocyte-macrophage colonies in vitro from the bone marrow (BM) of humans[4-6] and primates.[7] Recombinant human GM-CSF was also shown to stimulate eosinophil and multipotent progenitor cells[4] and have burst-promoting activity.[4-8] Mice[9] and nonhuman primates[7,10,11] have been used to evaluate the in vivo effects of recombinant GM-CSF. In a recent report,[12] the continuous intravenous administration of rhGM-CSF, both before and after autologous bone marrow transplantation, led to an accelerated neutrophil and platelet recovery.

In this report, the regulatory function of rhGM-CSF in hematopoietic regeneration is elucidated using an autologous bone marrow transplantation model with nonhuman primates. The protocol was designed to evaluate the regulatory effects of rhGM-CSF during early hematopoietic regeneration.

MATERIALS AND METHODS

Animals. Domestic-born male rhesus monkeys, *Macaca mulatta*, mean weight 7.6 (±0.5) kg, were housed in individual stainless steel cages in conventional holding rooms in an AALAC (American Association for Accreditation of Laboratory Animal Care) accredited animal facility. Monkeys were provided ten air changes/h of 100% fresh air, conditioned to 72°F (±2°F) with a relative humidity of 50% (±20%); provided commercial primate chow and tap water *ad libitum;* and maintained on a 12-hour light/dark full spectrum light cycle with no twilight. All research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

Transplantation. Anesthetized monkeys had bone marrow harvested one to two hours prior to irradiation by multiple iliac crest aspirations as previously described.[13] Low-density cells, used for transplantation, were isolated from harvested marrow by isopycnic centrifugation (400 × g, 30 min) using Lymphocyte Separation Medium (specific gravity 1.077, Litton Bionetics, Gaithersburg, MD). Bone marrow cells (5.0 × 10^7/kg) were administered intravenously one to two hours after exposure. During exposure, monkeys were restrained in a plexiglass chair and exposed bilaterally, receiving a midline tissue dose of 9.0 Gy (0.4 Gy/min) ^{60}Co gamma rays. Technical manipulations of the animals were performed with the monkeys under sedation (ketamine hydrochloride, 10 mg/kg, intramuscularly (IM)). After transplantation, the animals were clinically managed, as previously described,[13] with the administration of antibiotics (gentamycin sulfate, 3 mg/kg/d, IM) and platelets. Platelets, irradiated with 30 Gy, were administered when the count was below 50,000/mm^3. Red cells were not administered. Monkeys did not experience any infections during the study period.

Pump implantation. All monkeys were initially acclimated to rhesus monkey jackets (Alice King Chatham, Los Angeles) for a period of 2 weeks prior to the implantation of the pump. The jackets were worn continuously for an additional 2 weeks after the pump was

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implanted. Alzet miniosmotic pumps (2ML1, ALZA Corp, Palo Alto, CA), delivering 50,400 U/kg/d of rhGM-CSF over a period of seven days, were used in these experiments. Pumps were filled with either rhGM-CSF (n = 5) or saline (n = 2), and implanted between days 0 and 5. For surgical implantation of the pumps, monkeys were administered a general gas anesthesia: 0.5% to 5% halothane in a 65% N_2O-30% O_2 mixture. Pumps were implanted subcutaneously, along the midline of the back, caudal to the scapular crests. The skin was closed with 4/0 chromic gut placed in a continuous subcuticular suture pattern and bandaged. At the end of seven days, the pump was removed through the same surgical wound, flushed with a 50:50 hydrogen peroxide-sterile water solution and Betadine, and then packed with Betadine-soaked gauze and the animal lightly bandaged. The packing was changed on a daily basis for the next few days and was continually treated until healing was complete. All pump implantation sites healed within a week. The pump site was cultured for bacteria after pump removal and all bacterial cultures were negative.

Recombinant human GM-CSF. The rhGM-CSF used in these experiments was provided by Genetics Institute. A unit of activity for rhGM-CSF has been defined as the amount of GM-CSF needed to stimulate half of the maximal possible incorporation of ^{3}H-thymidine by CML peripheral blood myeloblasts.[7] The rhGM-CSF used in these studies had an activity of either 10^6 or 10^7 U/ml. A loading dose of 50,000 U/kg was administered intravenously on the day of pump implantation.

RESULTS

Recovery of Peripheral Blood Cells After Transplantation With or Without rhGM-CSF Infusion

The continuous subcutaneous administration of rhGM-CSF into autologous bone marrow transplanted monkeys (n = 5) led to an accelerated recovery of the peripheral blood (PB) neutrophils in comparison to control saline infused animals (n = 2) (Fig 1). Neutrophil levels recovered to 80% normal preirradiation values by day 20 in monkeys infused with rhGM-CSF, in comparison to 33% for control animals. The neutrophil recovery was also enhanced in contrast to the controls, reaching levels greater than 140% v 70% on day 31. The enhanced neutrophil production continued in the absence of rhGM-CSF. A consistent and sustained increase in neutrophil counts was observed in all treated monkeys. Treatment with rhGM-CSF led to a shortened time of neutropenia with a neutrophil count of >1,000/mm^3 on day 17 v day 20 for saline controls.

Platelet recovery in rhGM-CSF–infused monkeys was accelerated over that of controls, reaching 62% (220,000/mm^3) normal levels by day 28 v 20% (72,000/mm^3) for control monkeys on the same day. The early recovery reduced the period of thrombocytopenia and requirement for clinical platelet transfusions by at least two days.

Monocyte/macrophage recovery was enhanced but not accelerated. Monocyte levels reached 155% in rhGM-CSF–infused monkeys by day 20, in contrast to 60% recovery in control monkeys. The recovery of PB lymphocytes to normal levels was prolonged in rhGM-CSF infused animals. Lymphocyte levels in controls reached nearly 50% by day 40, while rhGM-CSF–infused monkeys were only at 12% of normal. Increased recovery of PB eosinophils was not observed in either the treated or untreated monkeys. Reticulocytes were not measured in this study; however, measured red blood cell parameters (hematocrit, RBC count, hemoglobin, and mean corpuscular volume) did not indicate any difference between the treated and untreated monkeys.

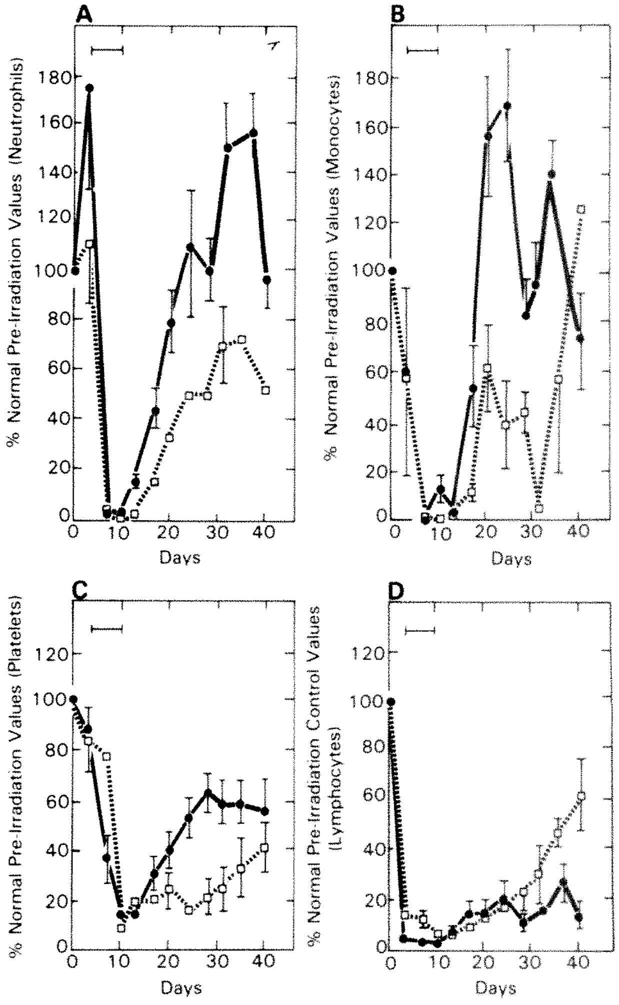

Fig 1. Peripheral blood cellular response, (A) neutrophils, (B) monocytes, (C) platelets, (D) lymphocytes, in monkeys after autologous bone marrow transplantation and then infused for seven days with rhGM-CSF (●) or Saline (□). Graphed as percent of mean normal preirradiation levels. A series of peripheral blood samples were drawn over a period of 3 to 4 weeks prior to initiating an experimental protocol to establish a hematologic baseline for each animal. Mean normal values for mature peripheral blood cells were determined from these pre-samples and identified as 100%. The mean (±SE) normal values of the monkeys used in this study are neutrophils, 4.2(±0.9) × 10^3/mm^3; platelets, 358,000 (±29,000)/mm^3; lymphocytes, 3.4(±0.4) × 10^3/mm^3; and monocytes, 0.4 ± 0.1 × 10^3/mm^3. The period of rhGM-CSF infusion is indicated with a bar.

Physiological Response of the Monkeys to the Infusion of rhGM-CSF

In comparison with controls (n = 2) and historical data (n = 10),[13] three of the monkeys infused with rhGM-CSF (n = 5) after irradiation and bone marrow (BM) transplantation experienced a severe weight loss (20% to 40%). The severity of the weight loss became apparent between 21 and 28 days, when saline control monkeys began to gain weight, and the rhGM-CSF–infused monkeys did not. The weight loss was not the result of anorexia or diarrhea since food intake and stools were comparable in both groups of monkeys. Two of the three monkeys experiencing a severe weight loss were euthanized (days 32 and 45) and necropsied. Pathology reports identified potential malabsorption associated with lymphangiectasia. The causes underlying this condition are currently under investigation. Both of these animals showed normal hematopoietic tissue.

DISCUSSION

Our objective in this study was to evaluate the regulatory properties of rhGM-CSF during early hematopoietic regeneration. The results demonstrate that a seven-day continuous subcutaneous treatment with rhGM-CSF during the early regenerative phase of hematopoiesis led to an accelerated recovery of PB neutrophils. The neutrophil response was also shown to be enhanced, attaining peak circulating counts 3 to 4 weeks after rhGM-CSF treatment. This continued neutrophil response is suggested to be the result of rhGM-CSF stimulating the regenerating BM to increase preferentially the size of the granulocyte-macrophage progenitor/stem cell pool. This increased progenitor/stem cell pool was thus responsible for the continued and elevated neutrophil output, even after rhGM-CSF was removed. Preliminary results for two of the rhGM-CSF–treated monkeys show that colony-forming unit granulocyte-macrophage (CFU-GM) activity in the BM attained peak activity (~140% of normal) on days 20 and 31. This was followed by a return to normal over the next 3 weeks. Observations using a hematopoietic suppression model also show that rhGM-CSF preferentially increases the pool of CFU-GM progenitor cells (unpublished observations).

Changes in the level of PB platelets after treatment with GM-CSF were not previously reported in either the normal murine[9] or primate models.[7] The recent report by Nienhuis and coworkers,[12] shows that GM-CSF treatment improves the recovery of platelets after transplantation. In our report, platelet recovery to a 50% level was also rapid, and reduced the time by two days that monkeys remained below a threshold for platelet transfusion. However, all treated monkeys demonstrated a consistent and sustained platelet recovery after treatment was terminated. In vitro results have suggested that megakaryocytes were stimulated by murine GM-CSF but that the stimulation required a higher dose of the factor than for CFU-GM progenitor cells.[11] The delivery dose used in these in vivo studies appeared sufficient for increased platelets in circulation. Whether rhGM-CSF has a direct effect on megakaryocyte progenitor cells or an indirect effect by increasing production of thrombopoietin or some other mechanism is uncertain.

The shortened period of neutropenia after bone marrow transplantation using GM-CSF was recently reported[12] and the results were comparable to our findings. In that study, GM-CSF was administered until the neutrophil count was at least >2,000/mm^3. However, circulating neutrophils were reduced by >50% upon termination of GM-CSF. This is in contrast to our results, which showed a sustained increase in neutrophils even after GM-CSF was removed.

We have therefore demonstrated that rhGM-CSF is an effective regulator of early hematopoietic regeneration, promoting an accelerated recovery of neutrophils and platelets even after GM-CSF was removed. The early effect of the GM-CSF was also long lasting, providing a sustained recovery of neutrophils and platelets. The potential clinical applications will include stimulation of hematopoietic regeneration following bone marrow aplasia, irradiation, cytotoxic therapy, or bone marrow transplantation.

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CONCISE REPORT

Tumor Necrosis Factor (TNF)-alpha but not TNF-beta Induces Secretion of Colony Stimulating Factor for Macrophages (CSF-1) by Human Monocytes

By W. Oster, A. Lindemann, S. Horn, R. Mertelsmann, and F. Herrmann

Tumor necrosis factor (TNF)-alpha has been identified as a major inducer of colony stimulating factor (CSF)-secretion by human vascular endothelial cells and fibroblasts. In the present study we assessed the capacity of TNFs to induce release of CSF-1 from highly purified peripheral blood monocyte preparations. Whereas monocytes do not accumulate CSF-1 messenger (m)RNA constitutively and consequently do not produce CSF-1 protein, CSF-1 mRNA and protein secretion became detectable, when monocytes were cultured in the presence of TNF-alpha, that was synergistically enhanced by interferon-gamma (IFN-gamma). However, under identical experimental conditions TNF-beta failed to induce monocyte CSF-1 synthesis. Cultures of monocytes in the presence of TNF-beta before addition of TNF-alpha abolished the CSF-1 inducing capacity of TNF-alpha, suggesting that TNF-beta may act as antagonist to TNF-alpha for CSF-1 production. These data point out a previously unrecognized function of TNF-alpha to modulate CSF-1 release by monocytes and demonstrate disparate biological properties of different TNF species in hematopoiesis.

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COLONY STIMULATING FACTOR (CSF)-1 (also referred to as M-CSF) has been genetically cloned and expressed[1] and biologically characterized.[2] CSF-1 specifically stimulates proliferation of monocyte/macrophage precursors,[2] induces selected functions of mature monocytes,[3] and is involved in the production of various biological mediators including interferon,[4] prostaglandin E,[5] interleukin 1,[6] CSF for granulocytes (G-CSF),[7] and TNF-alpha[8] by monocytes/macrophages. Recently, phorbolester-stimulated peripheral blood monocytes have been identified as a potent source of CSF-1.[9]

TNF-alpha, synthesized by activated monocyte/macrophage cells,[10] and TNF-beta, originating from stimulated T cells,[11] reveal a 36% identity and 51% homology in the overall amino acid sequence,[10] share common receptor binding domains,[12] and exert similar cytolytic and cytostatic effects on various tumor targets in vitro and in vivo.[13]

The roles of both TNF species have been described to extend to modulation of hematopoiesis, by sharing similar inhibitory effects on colony growth of granulocyte-macrophage, erythrocyte, and granulocyte-erythrocyte-megacaryocyte-macrophage precursors (CFU-GM, BFU-E, CFU-GEMM).[14-16] In addition, TNF-alpha has been shown to induce CSF secretion by human lung fibroblasts[17] and vascular endothelial cells[18] with molecular and biological characteristics of granulocyte-macrophage (GM)-CSF. So far there has been no evidence for disparate effects of TNF-alpha and TNF-beta on normal hematopoietic cells.

In the present study we assessed the capacity of both molecules to modulate CSF-1 expression and secretion by human monocytes, using Northern blot analysis and murine CFU-GM colony assays. Our data revealed that TNF-alpha but not TNF-beta transcriptionally regulates production of CSF-1 by human monocytes. TNF-alpha induced mRNA accumulation for CSF-1 is synergistically enhanced by the presence in culture of IFN-gamma and can be antagonized by TNF-beta.

MATERIALS AND METHODS

Preparation of monocytes and T cells. Peripheral blood derived mononuclear cells were isolated from leukapheresis residues of consenting healthy volunteer donors by Ficoll-Hypaque gradient (density 1.078 g/dL) centrifugation. T cells were recovered with AET (2-aminoethylisothiouronium bromide hydrobromide)-treated sheep RBCs (SRBCs) (5% vol/vol solution). Monocytes were separated by two sequential adherence steps of the SRBC-rosette negative fraction. Individual cell fractions, assessed by morphology (Wright Giemsa and alpha-naphthyl acetate esterase [ANAE] staining) and immunofluorescence analysis, using monoclonal antibodies (MoAb) to the T11, Mo2, and B1 antigens,[20] revealed preparations of >98% purity. Monocytes or T cells were plated at 10^6 cells/mL in RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin/streptomycin, L-glutamine and sodium pyruvate in plastic petri dishes (35 mm/well; Corning, New York). Recombinant human (rh) TNF-alpha or TNF-beta (kindly provided by Genentech, San Francisco, through R. Flener, Boehringer, Vienna) was added to cultures at 50 ng/mL (specific activity 5×10^7 U/mg or 1.2×10^8 U/mg, respectively). In selected experiments cultures received IFN-gamma (0.05 ng/mL; specific activity of IFN-gamma 4×10^7 U/mg; kindly provided by Genentech). In control cultures the addition of TNF-alpha, TNF-beta, and IFN-gamma was omitted. Cell-free supernatants were collected after 48 to 72 hours of culture. To neutralize the effects of carry-over of TNF preparations on growth of hematopoietic progenitor cells, TNF-monocyte conditioned media (TNF-MCM) and TNF-T cell conditioned media (TNF-TCM) were pretreated with MoAb to TNF-alpha or TNF-beta (kindly provided by Genentech) at concentrations sufficient to neutralize threefold more TNF-alpha or TNF-beta than was originally added. For RNA analysis cultures were performed for six to 24 hours or in selected experiments for up to 96 hours.

Northern blot analysis. Total RNA was prepared using the guanidium/cesium chloride method and denatured with glyoxal and dimethylsulfoxide as described previously[21] after quantification by spectralphotometry. Aliquots of each RNA were stained in minigels

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before denaturation with ethidium bromide to visualize 28 S and 18 S ribosomal RNA bands, in order to confirm quantification and integrity of the RNA samples. Identical amounts of total RNA (10 μg) were size-fractionated in 1.2% agarose gel electrophoresis, transferred to nylon base paper by capillary blot using 10 × SSC and baked for two hours at 18°C.[21] Specific c-DNA probe for M-CSF (1 kb Bgl I-PST I fragment cloned in PUC 18, kindly provided by P. Ralph, Cetus Corporation, Emeryville, CA) was radiolabeled as reported previously[22] with a specific activity of 2,5 to 3,5 × 10^8 cpm per μg DNA. Hybridization was performed in 50% formamide, 1% SDS, 5 × Denhardt's, 6 × SSPPE at 42°C for 18 hours. Filters, washed with 2 × SSC at 27°C, 0.1 × SSC, 1% SDS at 65°C and 0.1 × SSC at 27°C, were exposed to Kodak XAR5 with Dupont Cronex intensifying screens.

Murine CFU-GM colony assay. CFU-GM were assayed in a double layer agar culture system as previously described.[23] Underlayer (0.5 mL) were composed of 0.5% agar (Agar Noble, Difco, Detroit, IL) in Iscove's modified Dulbecco's minimum essential medium (IMDM) supplemented with L-glutamine, penicillin/streptomycin, 20% FCS and 5 × 10^{-5} mol/L 2-mercaptoethanol. As a potential source of CSF, media conditioned by monocytes (MCM) cultured for 48 to 72 hours in the presence or absence of TNF-alpha (50 ng/mL) and TNF-beta (50 ng/mL), respectively were added to the underlayers at 10% vol/vol final concentration. Overlayers (0.5 mL) were composed of 0.3% agar in the same medium and contained 5 × 10^4/mL murine (BDF, 1 mice) bone marrow cells (obtained from femures). CFU-GM were enumerated at day 7 and lineages of colonies were assessed by cytochemical in situ staining of whole agar cultures.[23] Naphtol-AS-D-chloroacetate esterase staining (CAE) detects granulocytic colonies, ANAE staining macrophage colonies.

RESULTS AND DISCUSSION

We have previously shown, that monocytes are a potential source of CSF-1 when induced with phorbol esters.[9] In the present study we investigated effects of TNF-alpha and TNF-beta on CSF-1 secretion by monocytes. Whereas monocytes do not transcribe CSF-1-gene constitutively (Fig 1, lane a) CSF-1 mRNA of 4.5 kb length became detectable, when monocytes were cultured in the presence of TNF-alpha for six hours (Fig 1, lane b) to 24 hours (not shown). In contrast, under identical conditions, TNF-beta failed to induce CSF-1 mRNA synthesis by monocytes (Fig 1, lane c); concentrations of TNF-beta >50 ng/mL (up to 500 ng/mL) and prolongation of incubation periods to >24 hours (up to 96 hours) did not affect CSF-1 processing (not shown). Autoradiograms exposed for as long as 14 days continued to show no signal for CSF-1 mRNA, when monocytes were incubated in the presence of TNF-beta. As in all cultures systems like the one used here, it is difficult to prove that the CSF-1 activity is actually released by monocytes and not by contaminating T cells. To determine if the effect of TNF-alpha on induction of CSF-1 release was on monocytes and not on possibly contaminating T cells, experiments were performed, in which the effects of TNFs on expression and secretion of CSF-1 by resting T cells were investigated.

Under experimental conditions that resulted in CSF-1 release by monocytes, T cells were unable to express or release CSF-1 in response to TNF-alpha (Fig 1, lane e). Since TNF-alpha and TNF-beta compete for binding to a common receptor domain[12] we investigated the possibility, whether TNF-beta acts as an antagonist to TNF-alpha for CSF-1 production by culturing monocytes sequentially in the presence of both factors. Treatment of monocytes with TNF-beta for six hours before addition of TNF-alpha abolished the potential of TNF-alpha to induce CSF-1 mRNA accumulation (Fig 2).

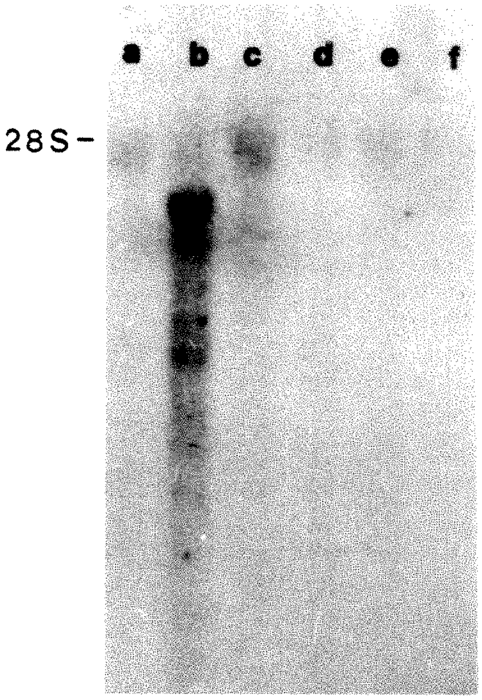

Fig 1. **Northern blot analysis probing CSF-1 cDNA. Total RNA derived from monocytes cultured for six hours in medium only (lane a), in medium containing 50 ng/mL TNF-alpha for six hours (lane b), or 50 ng/mL TNF-beta for six hours (lane c). RNA derived from T cells cultured for six hours in medium only (lane d), in the presence of 50 ng/mL TNF-alpha for six hours (lane e), or 50 ng/mL TNF-beta for six hours (lane f).**

Since IFN-gamma synergizes with TNFs in a variety of systems,[14-16] effects of IFN-gamma on TNF-alpha inducible CSF-1 mRNA accumulation was investigated. To this end, RNA of monocytes was probed with a CSF-1 c-DNA that

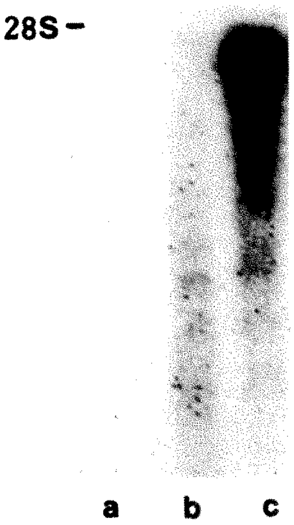

Fig 2. **Northern blot analysis probing CSF-1 cDNA. Total RNA derived from monocytes cultured for six hours in medium only (lane a), in medium containing 50 ng/mL TNF-alpha for six hours (lane c), and in medium containing 50 ng/mL TNF-beta for six hours before addition of 50 ng/mL TNF-alpha for a further six hours (lane b).**

Table 1. CSF-1 Secretion by Human Monocytes[*]

	Murine CFU-GM/5 × 10^4 Marrow Cells[*]					
	Monocyte (ANAE+)			Granulocyte (CAE+)		
Source	1	2	3	1	2	3
Control	11 ± 3[†]	12 ± 2	10 ± 2	13 ± 2	10 ± 2	10 ± 2
MCM	16 ± 2	9 ± 2	7 ± 3	0 ± 0	7 ± 2	3 ± 2
TNF-alpha MCM	116 ± 3	120 ± 2	123 ± 4	64 ± 7	76 ± 4	27 ± 7
TNF-beta MCM	18 ± 3	11 ± 2	12 ± 3	77 ± 6	70 ± 3	30 ± 4
TCM	12 ± 3	12 ± 2	13 ± 2	6 ± 2	7 ± 3	13 ± 4
TNF-alpha TCM	14 ± 4	13 ± 3	12 ± 3	7 ± 2	9 ± 3	9 ± 2
TNF-beta TCM	13 ± 2	17 ± 2	13 ± 3	10 ± 3	14 ± 2	13 ± 4

Abbreviation: Exp, experiment.

[*] Purified cell populations (monocytes or T cells) were cultured at 1 × 10^6/mL for 48 to 72 hours in the presence or absence of TNF-alpha or TNF-beta (50 ng/mL). Cell-free media were used as potential stimuli for murine CFU-GM at final concentrations of 10% vol/vol: Monocyte conditioned medium (MCM), in the presence of TNF-alpha (TNF-alpha MCM) or TNF-beta (RNF-beta MCM), T-cell conditioned medium (TCM) in the presence of TNF-alpha (TNF-alpha TCM) or TNF-beta (TNF-beta TCM).

[†] Colony numbers are means of triplicate CFU-GM assays (±SD).

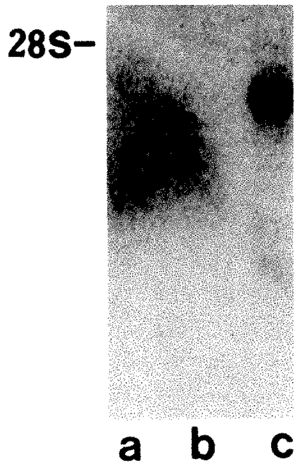

Fig 3. Northern blot analysis probing CSF-1 cDNA. Total RNA derived from monocytes cultured for 12 hours in medium containing 0.01 ng/mL TNF-alpha (lane a), 0.05 ng/mL IFN-gamma (lane b), or 0.01 ng/mL TNF-alpha plus 0.05 ng/mL IFN-gamma (lane c).

were cultured for 12 hours in the presence or absence of IFN-gamma and TNF-alpha in concentrations that had no effect on CSF-1 mRNA synthesis by their own. However, when cultures were performed in the presence of both factors, CSF-1 mRNA became detectable (Fig 3). To investigate whether increased levels of CSF-1 mRNA detected in TNF-alpha treated monocytes were accompanied by secretion of the biologically active protein, cell-free supernatants of monocytes, cultured in the presence of TNF-alpha (50 ng/mL) for 48 to 72 hours, were assayed in murine CFU-GM colony assays. Rh CSF-1 is known to stimulate only very small clusters of monocytes in agar culture of light density human marrow cells[2] but has the ability to induce the appearance of larger macrophage colonies on day 7 in a murine system. Rh GM-CSF is known to lack proliferative effects on adult or fetal murine hematopoietic cells[23] and rh G-CSF stimulates growth of murine granulocytic colonies only.[24] Thus, stimulation of murine macrophage colony growth distinguishes human CSF-1 from G- and GM-CSF activity. Murine colonies grown in double layer agar cultures were enumerated on day 7. Supernatants collected from unstimulated monocytes cultured for 48 to 72 hours contained negligible CSF-1 and G-CSF activity. Treatment of monocytes with TNF-alpha (50 ng/mL), but not TNF-beta, induced release of CSF-1 activity (Table 1). Our results point out a previously unrecognized function of TNF-alpha in modulating CSF-1 release by monocytes. Since monocytes lack CSF-1 mRNA constitutively, the level at which the upregulatory effects of TNF-alpha induced CSF-1 production take place is transcription.

In the context of the recent observation of induction of monocyte-TNF-alpha secretion by CSF-1[8] our present finding of a reversed induction sequence (ie, induction of CSF-1 secretion by TNF-alpha) suggests autocrine regulatory functions of both molecules. TNF-alpha induced CSF-1 release may stimulate proliferation of monocyte/macrophage precursor cells and augment the functional repertoire of mature monocytes in an autostimulatory manner.

In two of three experiments TNF-alpha MCM and TNF-beta MCM supported growth of granulocytic colonies as well (Table 1) suggesting the possibility that TNF-alpha not only induces secretion by monocytes of CSF-1 but also of G-CSF and that the inductive potential of TNF-beta for CSF-secretion is restricted to G-CSF.

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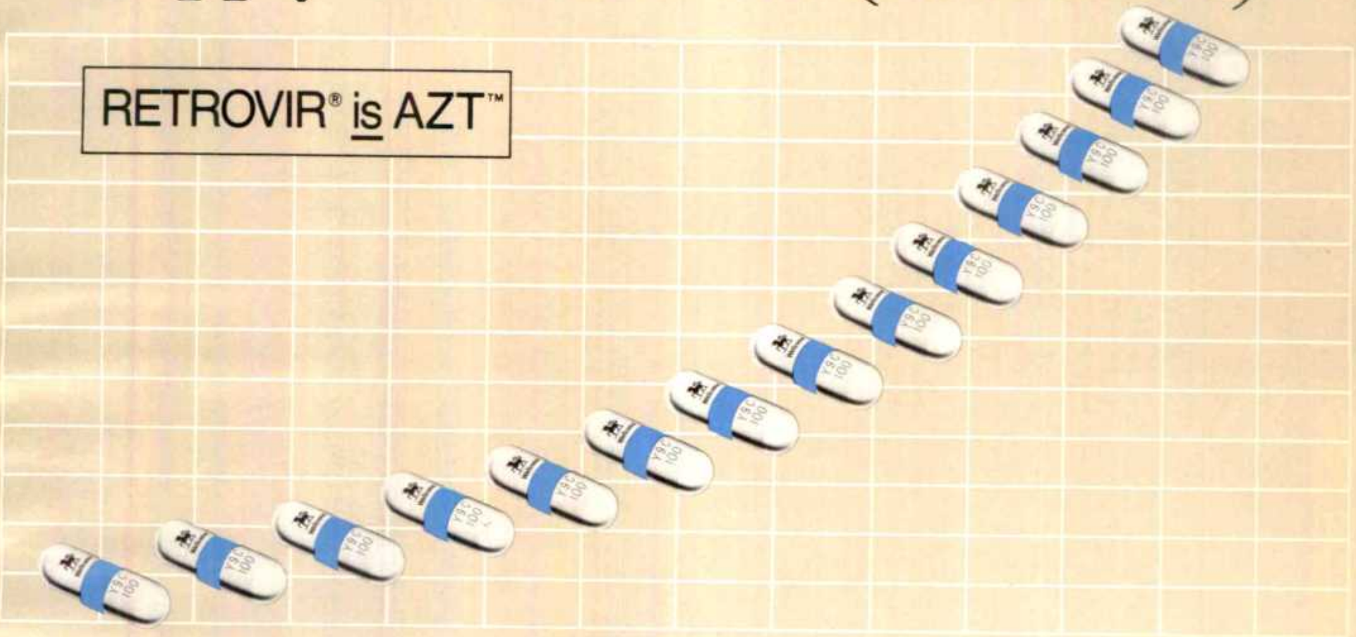

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INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper inducer) lymphocyte count of less than 200/mm^3 in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss (> 15 lbs or > 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 cells/mm^3 at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of < 750/mm^3. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count < 1000/mm^3 or hemoglobin < 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 µg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/TK$^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 µg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 µg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 µg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 µg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine; 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly related to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table:

	Pretreatment CD4 (T4) Levels			
	< 200/mm^3		> 200/mm^3	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia (< 750/mm^3)	47%	10%	10%	3%
Anemia (Hgb < 7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

		Pretreatment CD4 (T4) Levels			
		< 200/mm^3		> 200/mm^3	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	> 50%	55%	19%	40%	13%
Anemia	> 25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.
Cardiovascular: vasodilation.
Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.
Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.
Nervous: anxiety, confusion, depression, emotional lability, nervousness, syncope, loss of mental acuity, vertigo.
Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.
Skin: acne, pruritus, urticaria.
Special senses: amblyopia, hearing loss, photophobia.
Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of < 7.5 g/dL or reduction of > 25% of baseline) and/or significant granulocytopenia (granulocyte count of < 750/mm^3 or reduction of > 50% from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.

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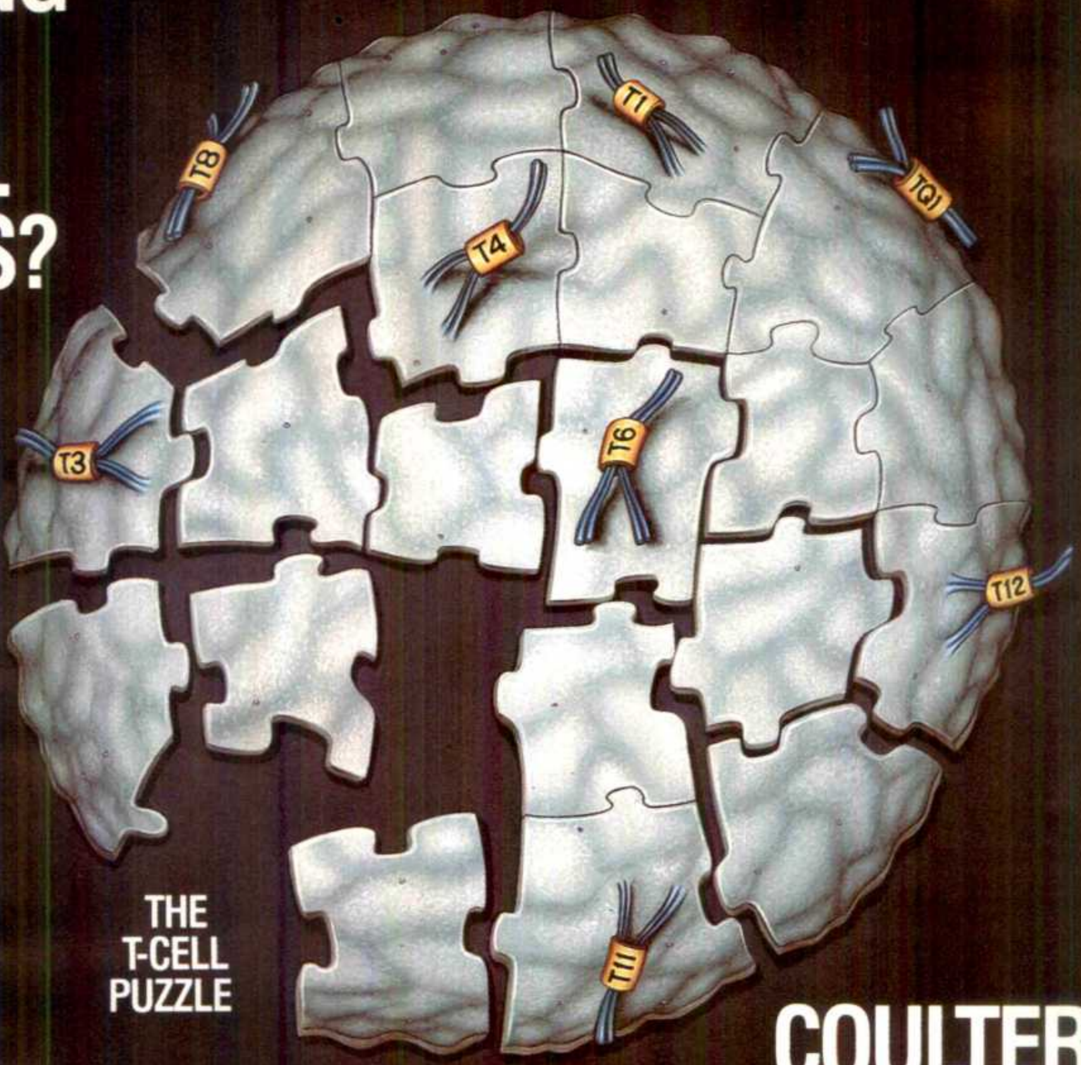

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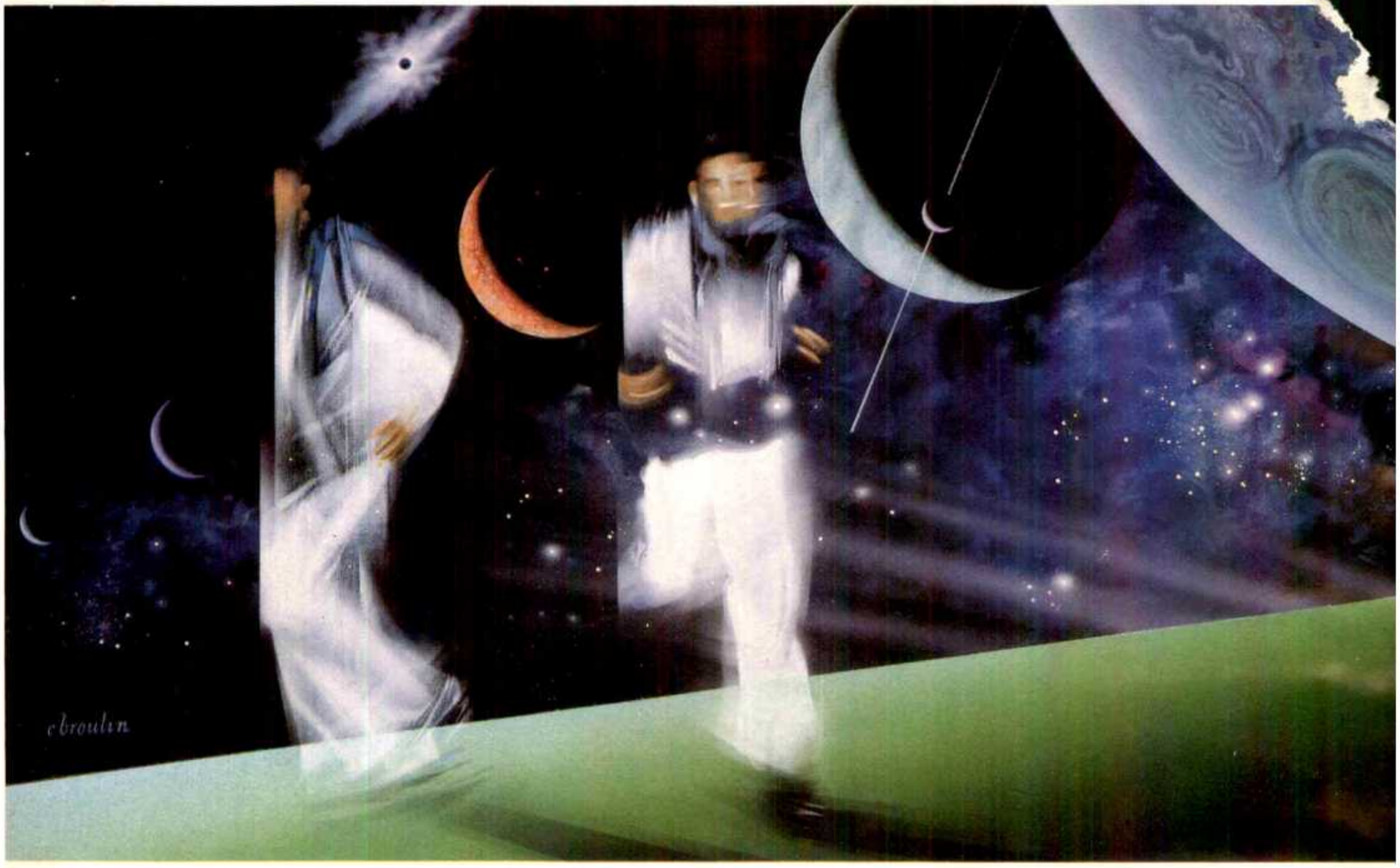

FORME ET PRESENTATION :
Lyophilisat et solution pour usage parentéral : Boîte unitaire

COMPOSITION :
Lyophilisat : 23,8 mg p. flacon.
Interféron alfa-2b 3 millions U.I.
ou 5 millions U.I. ou 10 millions U.I. ou 30 millions U.I.
Excipient : Sodium (hydrogénophosphate de) anhydre,
Phosphate monosodique monohydraté, Acide amino-acétique, Albumine humaine.
Solution : Eau pour usage parentéral : (1 ml).

SORT DU MEDICAMENT :
En sous-cutanée et intramusculaire, les pics sériques sont atteints en 8 et 6 h ; les demi-vies d'absorption en 4 et 3 h et les demi-vies d'élimination en 7 et 6 h.
En intraveineuse, le pic sérique est atteint à la 30^e minute (fin de perfusion), la demi-vie d'élimination est d'environ 1 h.
Quelle que soit la voie d'administration, il n'est pas retrouvé d'interféron alfa-2b dans les urines.

PROPRIETES :
Introna, dont le principe actif est l'interféron alfa-2b (interféron alpha, sous type 2) produit par génie génétique, présente ces propriétés qui ont été mises en évidence lors des études précliniques :
1. L'ACTIVITE ANTIPROLIFERATIVE : dose-dépendante.
2. L'ACTIVITE IMMUNOMODULATRICE.
3. L'ACTIVITE ANTIVIRALE.

INDICATIONS :
- Leucémie à tricholeucocytes.
- Mélanome disséminé.
- Sarcome de Kaposi associé au syndrome d'immuno-déficit acquis (SIDA).

CONTRE-INDICATIONS :
- Sensibilité à l'interféron alfa-2b ou à l'un des excipients (albumine humaine entre autres).
- GROSSESSE : en raison de la spécificité d'espèce, les études de tératogénèse et de toxicité fœtale chez l'animal n'ont pas de valeur prévisionnelle pour l'espèce humaine ; il en résulte que l'innocuité de l'interféron alfa-2b pendant la grossesse n'a pu être établie.

MISE EN GARDE :
- Une réaction allergique sévère doit faire interrompre le traitement immédiatement.
- Une surveillance électrocardiographique doit être assurée chez les patients ayant des antécédents cardiaques (infarctus du myocarde et/ou troubles du rythme) et ceux ayant reçu des substances cardiotoxiques.
- Une hypotension peut survenir durant le traitement et jusqu'à 48 h après son arrêt.
- Tout effet secondaire sévère doit conduire à modifier voire arrêter le traitement.

PRECAUTIONS :
- Un état d'hydratation satisfaisant doit être maintenu pendant la durée du traitement.
- Les analgésiques centraux, les hypnotiques et les sédatifs administrés simultanément doivent l'être avec prudence.
- L'innocuité de l'interféron alfa-2b n'a pas été établie ni chez l'enfant ni chez l'adolescent de moins de 18 ans.
- La surveillance biologique porte sur : NFS plaquettes, fonctions hépatique et rénale.

INTERACTIONS MEDICAMENTEUSES :
Incompatibilités majeures :
Aucune substance médicamenteuse ne doit être associée à l'Introna dans la seringue ou le flacon de perfusion.
Ne pas mélanger à des solutions glucosées.

EFFETS INDESIRABLES :
D'une façon générale, les effets indésirables ont un caractère réversible en 72 h et sont dose-dépendants :

Cliniques :
Le plus fréquent est le syndrome pseudo-grippal qui peut être prévenu ou traité par l'administration de paracétamol.
Les autres principaux effets indésirables observés sont :
- Les troubles du système nerveux central.
- Les modifications cardiovasculaires.
- Les troubles gastro-intestinaux.

Biologiques :
leuconeutropénie, thrombopénie, élévation des enzymes hépatiques, troubles de l'hémostase.

MODE D'EMPLOI ET POSOLOGIE :
- La reconstitution doit être effectuée immédiatement avant l' ploi avec les précautions d'asepsie d'usage.
  Prélever la dose appropriée pour injection sous-cutanée adjonction à 50 ml de soluté isotonique de NaCL pour admi tration intraveineuse (perfusion de 30 minutes).
- Aucune substance ne doit être associée à Introna dans seringue ou le flacon de perfusion.

Les posologies recommandées sont les suivantes :
- *Leucémies à tricholeucocytes :*
  2 millions d'Unités Internationales/m²/jour par voie sous-cutar 3 fois par semaine, 1 jour sur 2.
- *Mélanome disséminé :*
  5 à 10 millions d'Unités Internationales/m²/jour par voie s cutanée, 3 fois par semaine, 1 jour sur 2.
- *Sarcome de Kaposi associé au SIDA :*
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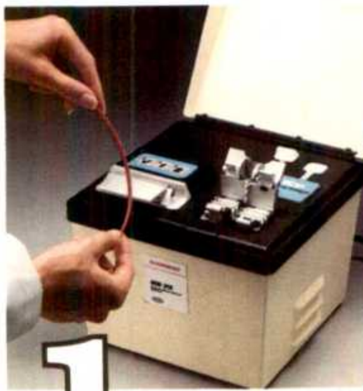

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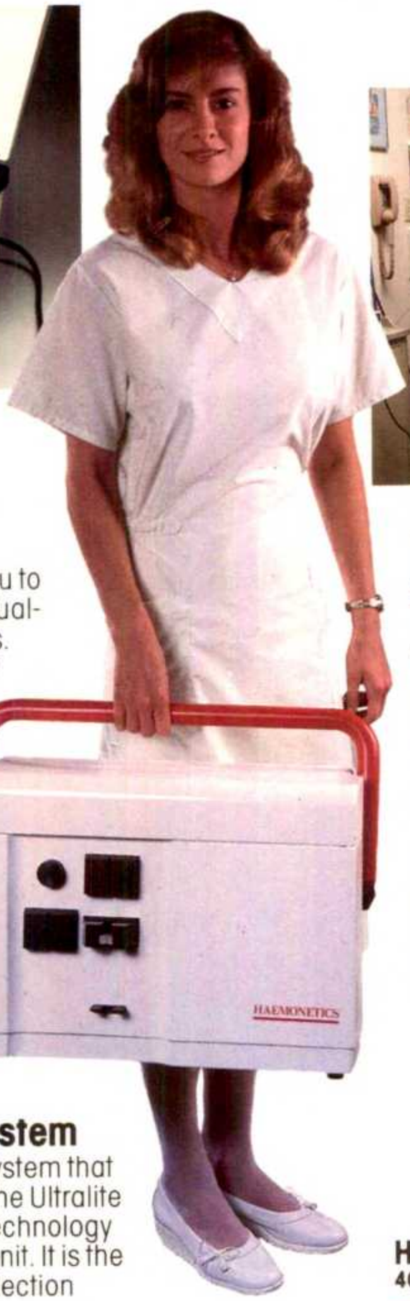

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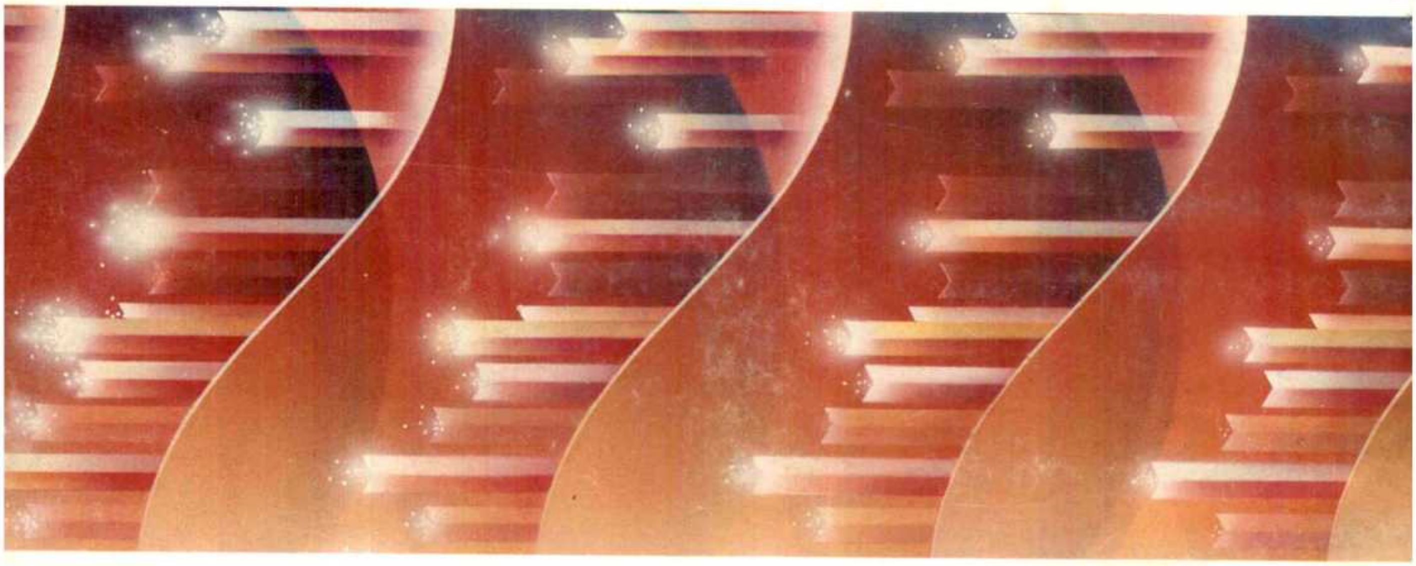

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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Please see brief summary of prescribing information on next page.
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CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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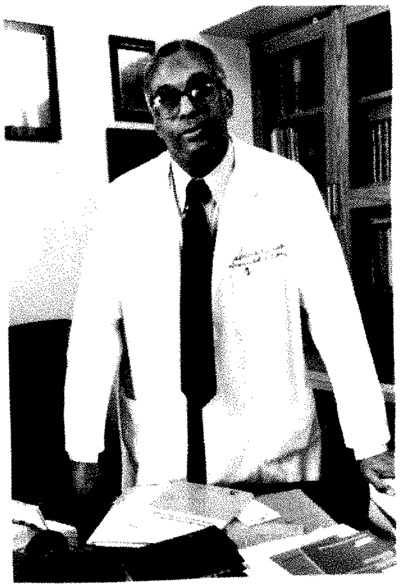

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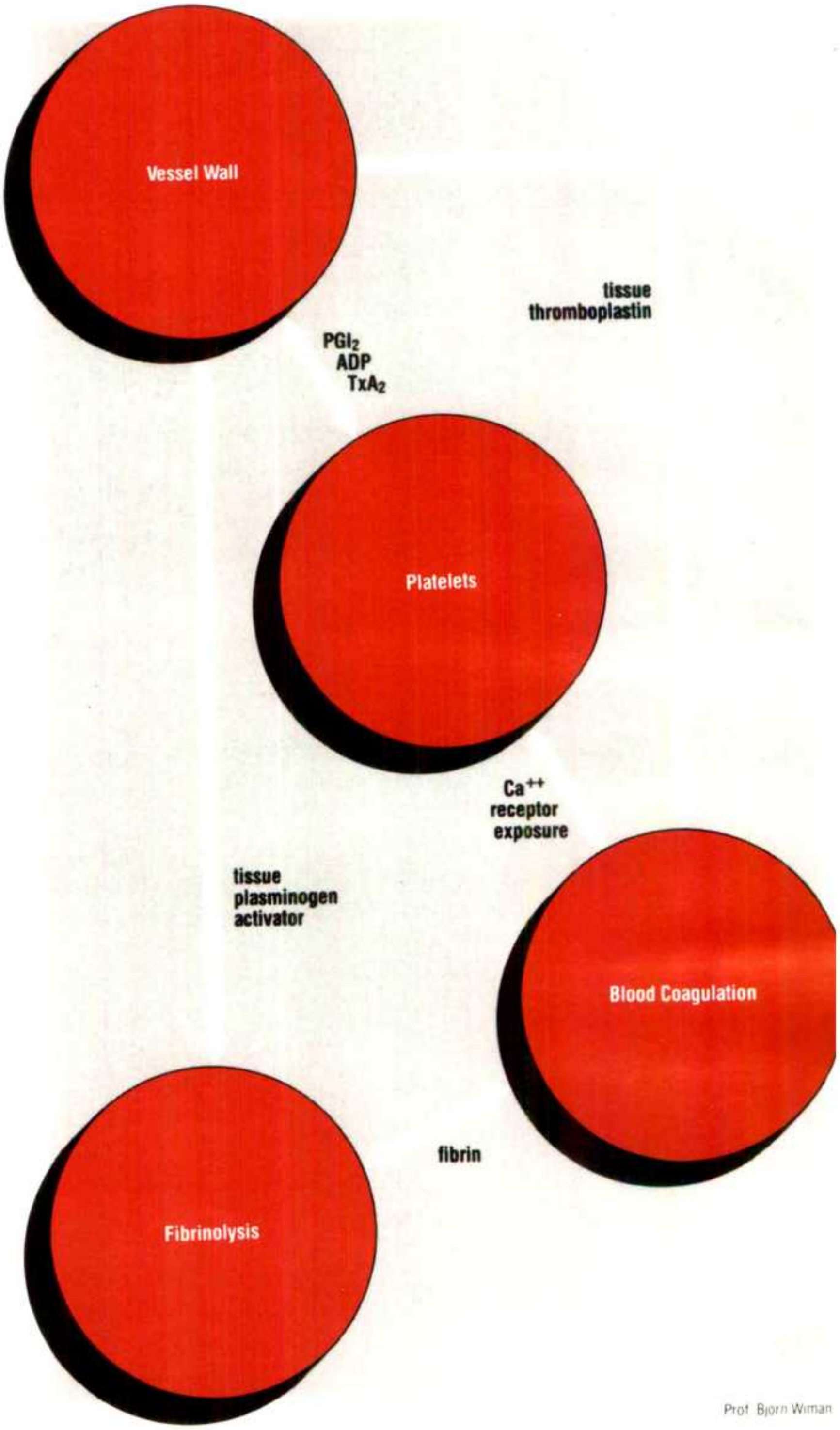

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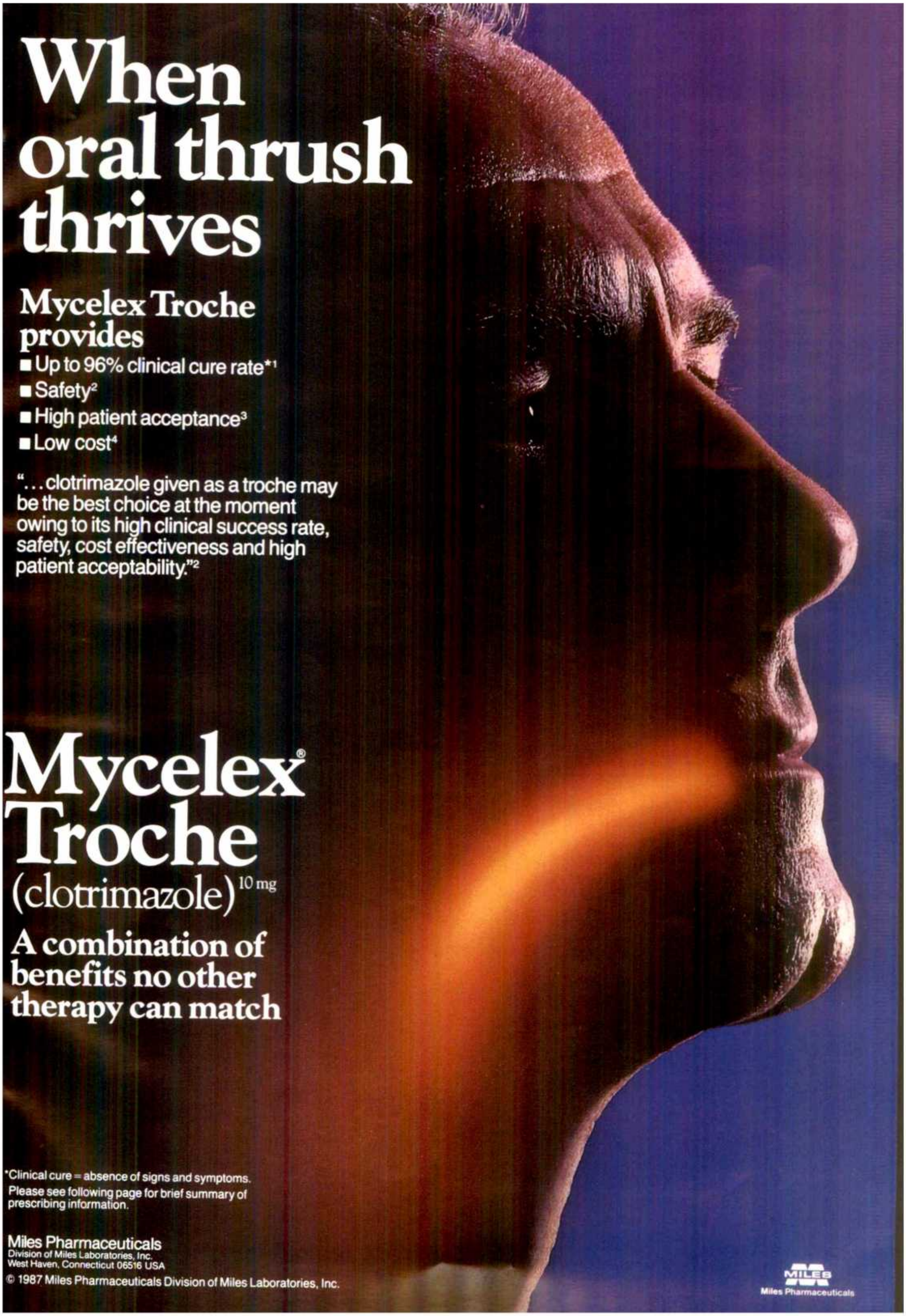

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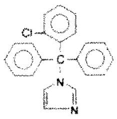

Chemical Formula:
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The troche dosage form is a large, slowly-dissolving tablet (lozenge) containing 10 mg of clotrimazole dispersed in dextrose, microcrystalline cellulose, povidone, and magnesium stearate.

Clinical Pharmacology
Clotrimazole is a broad-spectrum antifungal agent that inhibits the growth of pathogenic yeasts by altering the permeability of cell membranes. It exhibits fungicidal activity *in vitro* against *Candida albicans* and other species of the genus *Candida*. No single-step or multiple-step resistance to clotrimazole has developed during successive passages of *Candida albicans* in the laboratory.

After oral administration of a 10 mg clotrimazole troche to healthy volunteers concentrations sufficient to inhibit most species of *Candida* persist in saliva for up to three hours following the approximately 30 minutes needed for a troche to dissolve. The long term effective concentration of drug in saliva appears to be related to the slow release of clotrimazole from the oral mucosa to which the drug is apparently bound. Repetitive dosing at three hour intervals maintains effective salivary levels above the minimum inhibitory concentration of most strains of *Candida*. In another study the mean serum concentrations were 4.98 ± 3.7 and 3.23 ± 1.4 nanograms/ml at 30 and 60 minutes respectively.

Indications and Usage
Mycelex® Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications
Mycelex® Troches are contraindicated in patients who are hypersensitive to any of its components.

Warning
Mycelex® Troches are not indicated for the treatment of systemic mycoses.

Precautions
Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with pre-existing hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18 month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use
Safety and effectiveness of clotrimazole in children below the age of 3 years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions
Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).
Nausea and vomiting was reported in about one in twenty patients.

Overdosage
No data available.

Drug Abuse and Dependence
No data available.

Dosage and Administration
Mycelex® Troches are administered only as a lozenge that must be slowly dissolved in the mouth. The recommended dose is one troche five times a day for fourteen consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short term use, if possible.

How Supplied
Mycelex® Troches, white discoid, uncoated tablets are supplied in bottles of 70 and 140. Mycelex Troches are also available for institutional use in foil packages of 70 tablets. Each tablet will be identified with the following: Miles 095.

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References: 1. Yap B-S, Bodey GP. Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **2.** Quintiliani R, Owens NJ, Quercia RA, *et al.* Treatment and prevention of oropharyngeal candidiasis. *Am J Med* 77(4D):44-48, 1984. **3.** Data on file, Miles Pharmaceuticals. **4.** *Drug Topics Red Book, Annual Pharmacists' Reference.* Medical Economics Co., Oradell, NJ, 1987, pp. 397, 398, 411.

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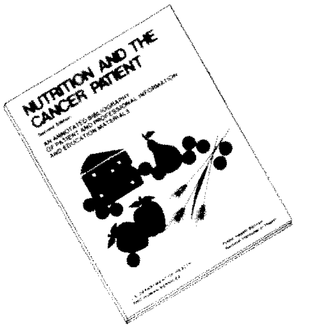

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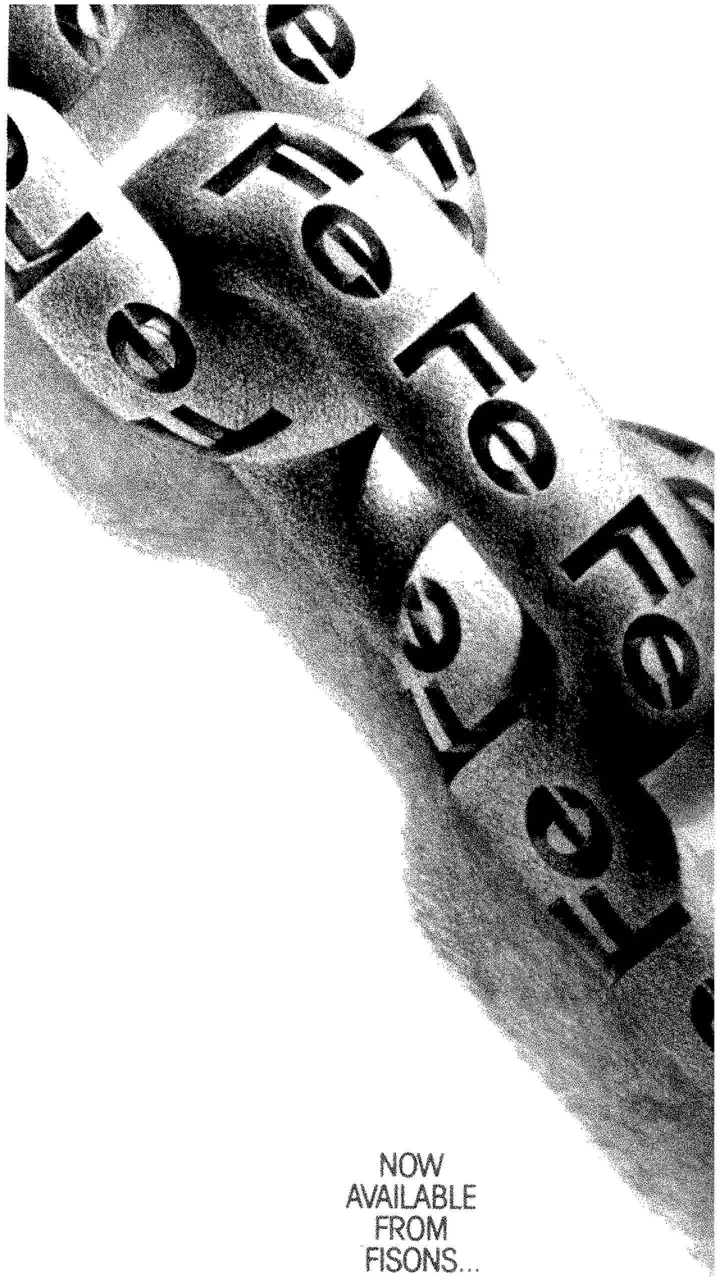

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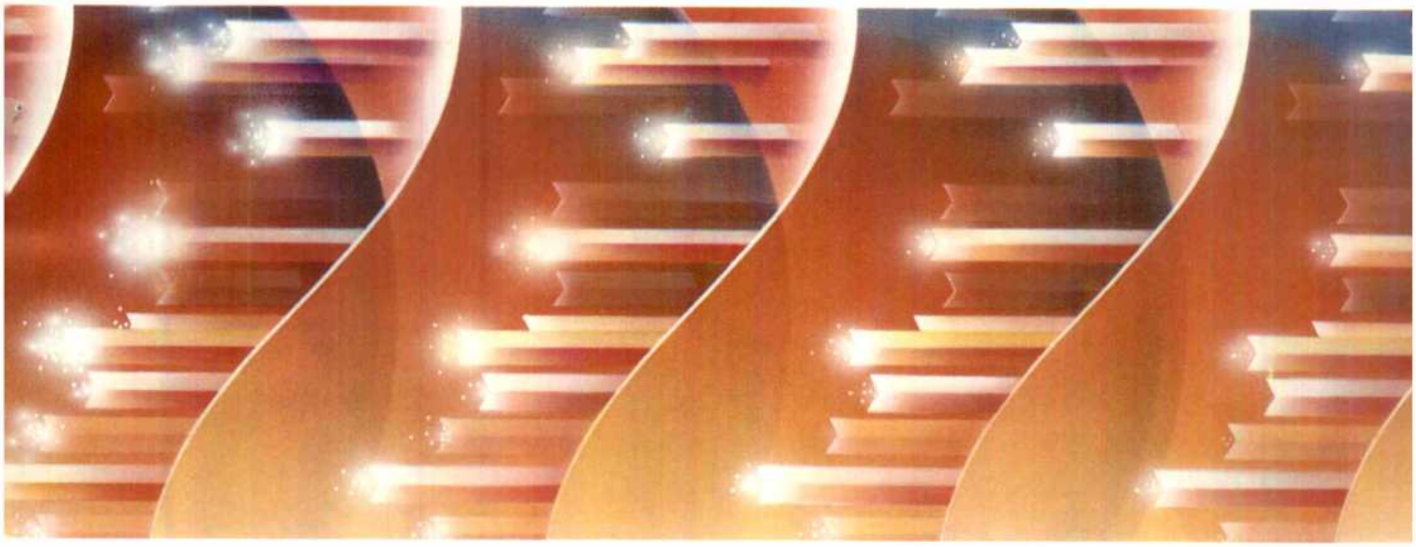
In the treatment of hemophilia A

A BREAKTHROUGH IN BIOTECHNOLOGY



NEW

MONOCLATE®

Factor VIII: C, Heat-Treated
Antihemophilic Factor
(Human)

**Now available from
Armour Pharmaceutical Company**

Please see next page for brief summary of prescribing information

ANTIHEMOPHILIC FACTOR (HUMAN)
MONOCLATE®
FACTOR VIII:C, HEAT-TREATED

BRIEF SUMMARY

DESCRIPTION: Antihemophilic Factor (Human), MONOCLATE® Factor VIII:C, Heat-Treated is a sterile, stable, lyophilized concentrate of Factor VIII:C with reduced amounts of Factor VIII:R and purified of extraneous plasma-derived protein by use of affinity chromatography. A murine monoclonal antibody to Factor VIII:R is used as an affinity ligand to first isolate the Factor VIII complex. Factor VIII:C is then dissociated from VIII:R, recovered, formulated and provided as a sterile lyophilized powder. The product as formulated contains Albumin (Human) as a stabilizer, resulting in a concentrate with specific activity between 5 and 10 units/mg of total protein. In the absence of this added Albumin (Human) stabilizer, specific activity has been determined to exceed 3000 units/mg of protein. MONOCLATE® has been prepared from pooled human plasma and is intended for use in therapy of classical hemophilia (Hemophilia A).

This product has been subjected to a heat-treatment process during its manufacture in order to reduce the risk of viral transmission, although no procedure has been shown to be totally effective in removing viral infectivity from coagulant factor products.

MONOCLATE® is a highly purified preparation of Factor VIII:C. When stored as directed, it will maintain its labeled potency for the period indicated on the container and package labels.

Upon reconstitution, a clear, colorless solution containing 50 to 150 times as much Factor VIII:C as does an equal volume of plasma is obtained.

Each vial contains the labeled amount of antihemophilic factor (AHF) activity as expressed in terms of International Units of antihemophilic activity. One unit of antihemophilic factor activity is equivalent to that quantity of AHF present in one mL of normal human plasma. When reconstituted as recommended the resulting solution contains approximately 300 to 450 millimoles of sodium ions per liter and has 2 to 3 times the tonicity of saline. It contains approximately 2-5 millimoles of calcium per liter contributed as calcium chloride, approximately 1-2% Albumin (Human), 0.8% mannitol, and 1.2 mM histidine. The pH is adjusted with hydrochloric acid and/or sodium hydroxide. MONOCLATE® also contains trace amounts (less than 50 ng per 100 AHF activity units) of mouse protein (see CLINICAL PHARMACOLOGY).

MONOCLATE® is to be administered only intravenously.

CLINICAL PHARMACOLOGY: Factor VIII:C is the coagulant portion of the Factor VIII complex circulating in plasma. It is noncovalently associated with the Factor VIII:R protein responsible for von Willebrand factor activity. These two proteins have distinct biochemical and immunological properties and are under separate genetic control. Factor VIII:C acts as a cofactor for Factor IX to activate Factor X in the intrinsic biological amplification of plasma enzymic proteins concerned with blood coagulation. Hemophilia A, an hereditary disorder of blood coagulation due to decreased levels of Factor VIII:C, results in profuse bleeding into joints, muscles or internal organs as a result of a trauma. Antihemophilic Factor (Human), MONOCLATE®, Factor VIII:C, Heat-Treated, provides an increase in plasma levels of AHF, thereby enabling temporary correction of hemophilia A bleeding.

Clinical evaluation of MONOCLATE®, Factor VIII:C heat-treated product for its half-life characteristics in hemophilic patients showed it to be comparable to other commercially available Factor VIII products. An estimated half-life of 15 hours was obtained.

Evidence of the capability of the manufacturing processes used in the production of Antihemophilic Factor (Human), MONOCLATE®, Factor VIII:C, Heat-Treated to reduce viral bioburden was obtained in inactivation studies involving the addition of known quantities of virus to cryoprecipitate. Viruses used were human immunodeficiency virus (HIV), Sindbis virus, vesicular stomatitis virus (VSV), and pseudorabies virus (PsRV).

With the assay methods used, HIV was reduced by ≧ 7.08 and ≧ 9.1 logs (in two experiments); model virus experiments demonstrated Sindbis virus reductions of ≧ 8.21 and ≧ 9.95 logs, VSV reduction of 7.51 logs, and PsRV reduction of ≧ 6.85 logs.

A total of 5 lots of MONOCLATE® were administered to a cohort of 11 patients who had not previously received any coagulation factor concentrates. The study showed no evidence of nonA, nonB hepatitis transmission as determined by bi-weekly ALT measurements over observation periods ranging from 4 months to 6 months.

Although MONOCLATE® contains trace amounts of mouse protein (less than 50 ng per 100 AHF activity units), only 1 of 18 patients treated on multiple occasions and followed for between 6 and 14 months showed evidence of antibody to mouse protein above baseline. In that patient the antibody level did not change after the second month of treatment and was not associated with any clinical adverse reaction. Pre-existing antibody to mouse protein was detected in some patients prior to MONOCLATE® administration: in the assay used, people with rheumatoid factor may test falsely positive and other proteins may also show cross-reactivity.

INDICATIONS AND USAGE: Antihemophilic Factor (Human) MONOCLATE®, Factor VIII:C, Heat-Treated is indicated for treatment of classical hemophilia (Hemophilia A). Affected individuals frequently require therapy following minor accidents. Surgery, when required in such individuals, must be preceded by temporary corrections of the clotting abnormality. Presurgical correction of severe AHF deficiency can be accomplished with a small volume of MONOCLATE®.

MONOCLATE® is not effective in controlling the bleeding of patients with von Willebrand's disease.

CONTRAINDICATIONS: Known hypersensitivity to mouse protein is a contraindication to Antihemophilic Factor (Human), MONOCLATE®, Factor VIII:C, Heat-Treated.

WARNINGS: MONOCLATE® is prepared from pooled units of human plasma which may contain the causative agents of hepatitis B, nonA, nonB hepatitis, acquired immunodeficiency syndrome (AIDS) and other viral diseases. Each unit of plasma used in the production of MONOCLATE® has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) and antibody to human immunodeficiency virus (HIV) by FDA approved tests, and has been shown to have ALT levels not exceeding two times the upper limit of normal. Additional screening procedures used to eliminate high risk plasma donors, and the purification techniques and heat-treatment step used in the manufacturing process, are all designed to reduce the risk of transmitting viral infection. However, testing methods presently available are not sensitive enough to detect all units of potentially infectious plasma, and treatment methods have not been shown to be totally effective in eliminating any of the aforementioned viral diseases from this product. Accordingly, the benefits and risks of treatment with this product should be carefully assessed prior to use.

Individuals who have not received multiple infusions of blood or plasma products are likely to develop signs and/or symptoms of some viral infections, especially nonA, nonB hepatitis as shown by recent data, when treated with coagulant factor products. As indicated under CLINICAL PHARMACOLOGY, however, a group of such patients treated with MONOCLATE® did not demonstrate signs or symptoms of nonA, nonB hepatitis over observation periods ranging from 4 months to 6 months.

PRECAUTIONS: General — Most Antihemophilic Factor (Human) concentrates contain naturally occurring blood group specific antibodies. However, the processing of MONOCLATE® significantly reduces the presence of blood group specific antibodies in the final product. Nevertheless, when large or frequently repeated doses of product are needed, patients should be monitored by means of hematocrit and direct Coombs tests for signs of progressive anemia.

Formation of Antibodies to Mouse Protein — Although no hypersensitivity reactions have been observed, because MONOCLATE® contains trace amounts of mouse protein (less than 50 ng per 100 AHF activity units), the possibility exists that patients treated with MONOCLATE® may develop hypersensitivity to the mouse proteins.

Information for Patients

Patients should be informed of the early signs of hypersensitivity reactions including hives, generalized urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis, and should be advised to discontinue use of the product and contact their physician if these symptoms occur.

Pregnancy Category C — Animal reproduction studies have not been conducted with Antihemophilic Factor (Human), MONOCLATE® Factor VIII:C, Heat-Treated. It is also not known whether MONOCLATE® can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. MONOCLATE® should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS: Products of this type are known to cause allergic reactions, mild chills, nausea or stinging at the infusion site.

HOW SUPPLIED: MONOCLATE® is supplied in a single dose vial with diluent, sterile needles for reconstitution and withdrawal, winged infusion set, and alcohol swabs. I.U. activity is stated on the label of each vial.

CAUTION: Federal (U.S.A.) law prohibits dispensing without prescription.

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ARMOUR PHARMACEUTICAL COMPANY
Kankakee, Illinois 60901

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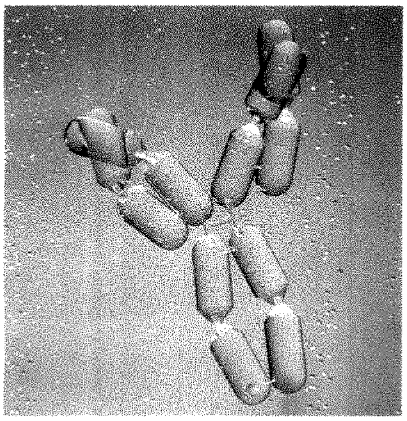

GAMMAGARD®
Immune Globulin
Intravenous (Human)

GAMMAGARD® IGIV offers all these benefits.

DEAE Sephadex® purification process provides high degree of functional activity.[1]

- Indicated in Idiopathic Thrombocytopenic Purpura (ITP)
  - 100% response in a clinical study[2]
- Indicated in immunodeficiencies
  - High titers against a wide range of pathogens

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For more information, see your sales representative or contact Baxter Healthcare Corporation, Hyland Division, 444 West Glenoaks Blvd., Glendale, CA 91202 USA, 800.423.2090.

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[1] Landsperger WJ, Gallagher MJ, Branch DR. Biological activity of intravenous immunoglobulins. *The Lancet* May 2, 1987. 1035

[2] Sixteen patients (children and adults) with acute or chronic ITP were studied at New York Hospital—Cornell Medical Center. Childhood ITP may resolve spontaneously without treatment.

Hyland Division

Baxter

DIRECTION INSERT

mmune Globulin Intravenous (Human)
GAMMAGARD®

ESCRIPTION

Immune Globulin Intravenous (Human), GAMMA-ARD® * is a sterile, dried, highly purified preparation f immunoglobulin which is derived from the cold ethanol actionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with e appropriate volume of diluent, this preparation con-ains approximately 50 mg of protein per mL, of which t least 90% is gamma globulin. The reconstituted pro-uct contains approximately 0.15M sodium chloride and as a pH of 6.8±0.4. Stabilizing agents are present in the ollowing maximum amounts: 20 mg/mL glucose, 2 g/mL PEG, 0.3M glycine, and 3mg/mL human albumin.

The manufacturing process for Immune Globulin In-avenous (Human), GAMMAGARD, isolates gamma lobulin without additional chemical or enzymatic modifica-on and the Fc portion is maintained intact. Immune lobulin Intravenous (Human), GAMMAGARD contains all e immunoglobulin G antibody activities which are pre-ent in the donor population. On the average, the distribu-on of IgG subclasses present in this product is the same s is present in normal plasma.[1] Immune Globulin Intra-enous (Human), GAMMAGARD contains only trace mounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD ontains no preservative.

This product has been prepared from large pools of uman plasma which was taken only from plasma units ound to have normal levels of alanine aminotransferase ALT). Each unit of plasma used in the manufacture of this roduct has been found to be nonreactive for HBsAg anti-ody by an FDA approved test.

LINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD ontains a broad spectrum of IgG antibodies against acterial and viral agents that are capable of opsonization nd neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infu-ion of Immune Globulin Intravenous (Human), GAMMA-ARD. It has been shown that IgG is distributed relatively apidly between plasma and extravascular fluid until approx-mately half of the total body pool is partitioned in the ex-ravascular space. A rapid initial drop in serum levels is, herefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum roteins.[2,3] Studies show that the half-life of Immune lobulin Intravenous (Human), GAMMAGARD is approx-mately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG an vary considerably from person to person, however. In articular, high concentrations of IgG and hyper-metabolism ssociated with fever and infection have been seen to coin-ide with a shortened half-life of IgG.[2,3,4,5]

NDICATIONS AND USAGE

mmunodeficiencies

Immune Globulin Intravenous (Human), GAMMAGARD s efficacious in the treatment of primary immunodeficient states, such as: congenital agammaglobulinemias, com-non variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5] Immune lobulin Intravenous (Human), GAMMAGARD is especial-y useful when high levels or rapid elevation of circulating amma globulins are desired or when intramuscular in-ections are contraindicated (e.g., small muscle mass).

diopathic Thrombocytopenic Purpura (ITP)

When a rapid rise in platelet count is needed to control leeding or to allow a patient with ITP to undergo surgery, he administration of Immune Globulin Intravenous Human), GAMMAGARD should be considered. The efficacy f Immune Globulin Intravenous (Human), GAMMAGARD as been demonstrated in a clinical study involving six-een patients (twelve adults and four children) diagnosed with acute or chronic Idiopathic Thrombocytopenic Pur-ura (ITP). Each of the sixteen patients (100%) demonstrated an acute, clinically signficant rise in platelet count (platelet count greater than $40{,}000/mm^3$) following the administration of Immune Globulin Intravenous (Human), GAMMAGARD.

Ten of the sixteen patients (62%) exhibited a clinically significant rise in platelet count after only one 1 g/kg infusion; four patients (25%) exhibited this result after only two 1 g/kg infusions; and two patients exhibited this result after more than two 1 g/kg infusions. The rise in platelet count is generally rapid, occurring within 5 days. The rise, however, is transient and should not be considered curative. Platelet rises most often lasted 2 to 3 weeks, with a range of 12 days to 6 months. It should be noted that childhood ITP may resolve spontaneously without treatment.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 µg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever, urticaria, flushing and nausea may occasionally occur. The incidence of these reactions directly attributable to the infusion of Immune Globulin Intravenous (Human), GAMMAGARD during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

During the clinical study of this product for treatment of ITP, the only side effect reported was headache which occurred in 12 of 16 patients. Oral antihistamines and analgesics alleviated the headache and were used as pre-treatment for those patients requiring additional IGIV therapy. The remaining four patients did not report any side effects and did not require pre-treatment.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Immunodeficiencies

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response. The minimum serum concentration of IgG necessary for protection has not been established.

Idiopathic Thrombocytopenic Purpura (ITP)

For patients with acute or chronic Idiopathic Thrombocytopenic Purpura (ITP), a dose of 1 g/kg is recommended. The need for additional doses can be determined b clinical response and platelet count. Up to three doses m be given on alternate days if required.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg/H be used. If infusion at this rate causes the patient n distress, the administration rate may be gradually increase but should not exceed 4 mL/kg/Hr.

A rate of administration which is too rapid may caus flushing and changes in pulse rate and blood pressur Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGAR should be administered as soon after reconstitution a possible. Administration should begin not more than 2 hour after reconstitution.

The reconstituted material should be at room temperatur during administration.

Parenteral drug products should be inspected visual for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure th administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGAR is supplied in either 2.5 g or 5.0 g single use vials. Eac vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Wate for Injection, USP, a transfer device and an administratio set which contains an integral airway and a 15 micron filte

Storage

Immune Globulin Intravenous (Human), GAMMAGAR is to be stored at a temperature not to exceed 25°C (77°F Freezing should be avoided to prevent the diluent bott from breaking.

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1. Unpublished data in files of Baxter Healthcare Corporation, Hyland Division.
2. Waldmann TA, Storber W: Metabolism of immunoglobulins. *Prog Allergy 13*: 1-110, 1969
3. Morell A, Riesen W: Structure, function and catabolis of immunoglobulins in *Immunohemotherapy.* Nydegger U (ed), London, Academic Press, 1981, pp 17-26
4. Stiehm ER: Standard and special human immune seru globulins as therapeutic agents. *Pediatrics* 63:301-319, 197
5. Buckley RH: Immunoglobulin replacement therapy: indications and contraindications for use and variable Ig levels achieved in *Immunoglobulins: Characteristics ar Use of Intravenous Preparations.* Alving BM, Finlayson J (eds), Washington, D.C., U.S. Department of Health ar Human Services, 1979, pp 3-8
6. Burks AW, Sampson HA, Buckley RH: Anaphylactic reactions after gammaglobulin administration in patients wi hypogammaglobulinemia: Detection of IgE antibodies IgA. *N Eng J Med 314*:560-564, 1986

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Inquiries:
(800) 423-2090

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*Manufactured under U.S. Patent No. 4,439,421

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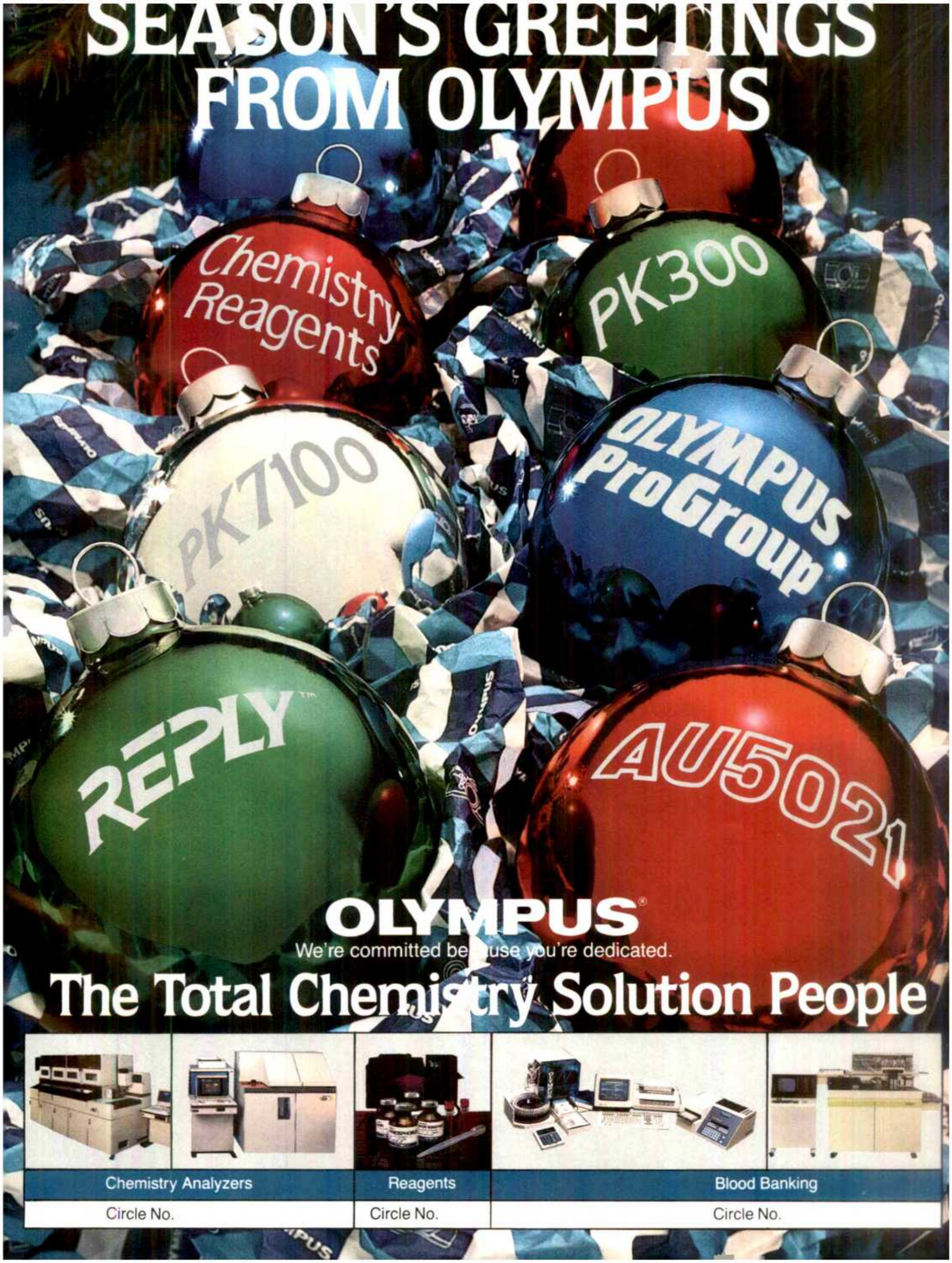

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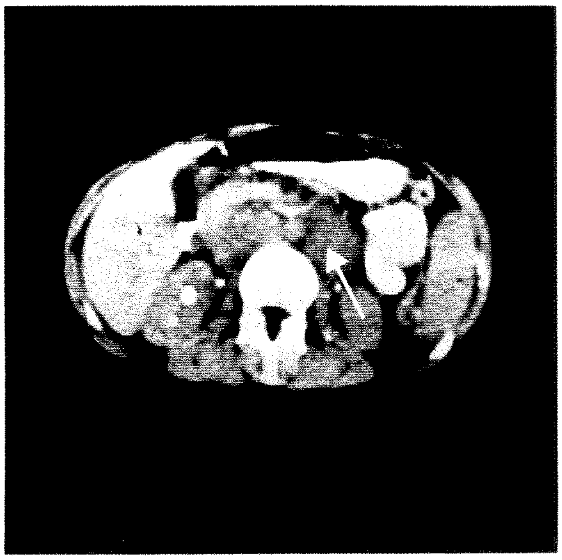
Proven Effective in Non-Hodgkin's Lymphoma

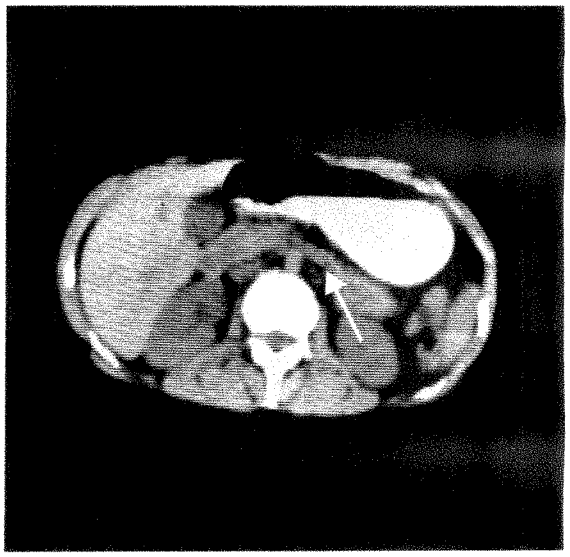
The newer Blenoxane containing combinations resulted in high complete response rates and extended disease-free survival.[1-7]

Abdominal/Pelvic CT
February 25



Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al*: Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al*: Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen S *et al*: Combination chemotherap advanced diffuse histiocytic lymphoma with the six-drug COl BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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sterile bleomycin sulfate)

# of Chemotherapy Regimens in Non-Hodgkin's Lymphoma

Since Blenoxane is virtually non-myelosuppressive, continuing therapy with certain Blenoxane containing chemotherapy regimens e.g. BACOP) can prevent tumor regrowth between cycles of other chemotherapy regimens while allowing for bone marrow ecovery.[*2,3,4,6]

Blenoxane can produce, with cumulative doses, a syndrome of pulmonary fibrosis. It is usually dose-related, being seen infrequently n patients whose total dose of Blenoxane is less than 400 units.

Efficacy . . . Yesterday, Today, Tomorrow

Blenoxane®
(sterile bleomycin sulfate)

M-BACOD

1. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
2. Anderson KC, Skarin AT, Rosenthal DS, *et al:* Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al:* Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

Blenoxane® (sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and ulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.
2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

SUPPLY: Each vial contains 15 units of Blenoxane as sterile bleomycin sulfate. NDC 0015-3010-20

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AIDS
UPDATE

Reports in the New England Journal of Medicine confirm the early promise for many patients with AIDS and other serious HIV infections...

RETROVIR® (zidovudine)

Helps patients
live longer, live better

RETROVIR® is AZT™

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR®

(zidovudine)

Helps patients live longer, live better

New England Journal of Medicine reports

"[RETROVIR] AZT administration was therefore associated with a fourfold-to-sixfold reduction in the mortality rates in the study population at nine months."[1]

Patients live longer

A 90% survival rate was reported for AIDS and ARC patients treated with RETROVIR after one year—*v* 50% in a similar group of untreated patients[1,2]

Patients live better

Improves patients' general health and quality of life[1]

- Significantly reduces symptoms related to HIV infection
- Many patients can resume productive lives
- Reduces risk and severity of opportunistic infections
- Improves immune status

Improves patients' cognitive status[3,4]

- Produced statistically significant improvements in long-term memory, motor function, concentration, and information-processing ability
- RETROVIR crosses the blood-brain barrier

IMPROVING LIVES THRO
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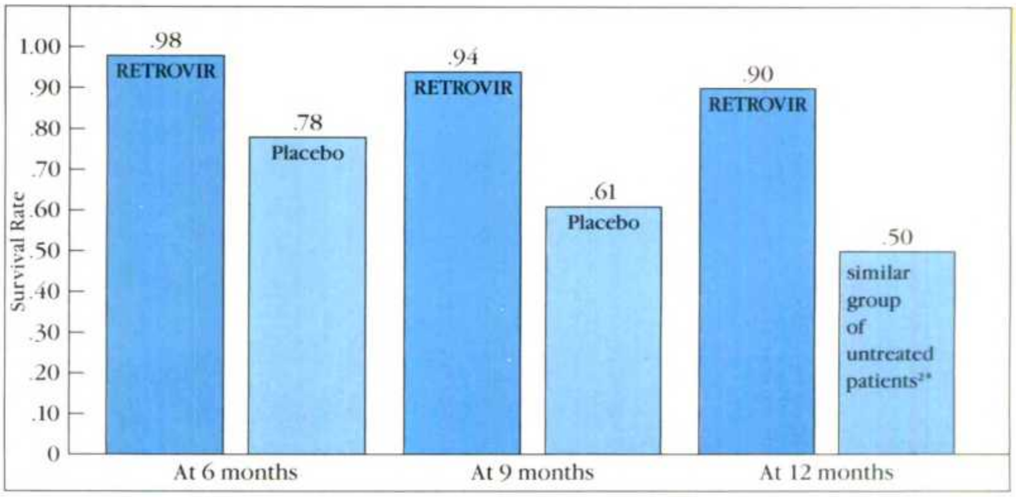
Helps patients live longer

Survival rates for AIDS/ARC patients combined at 6, 9, and 12 months[1,2]

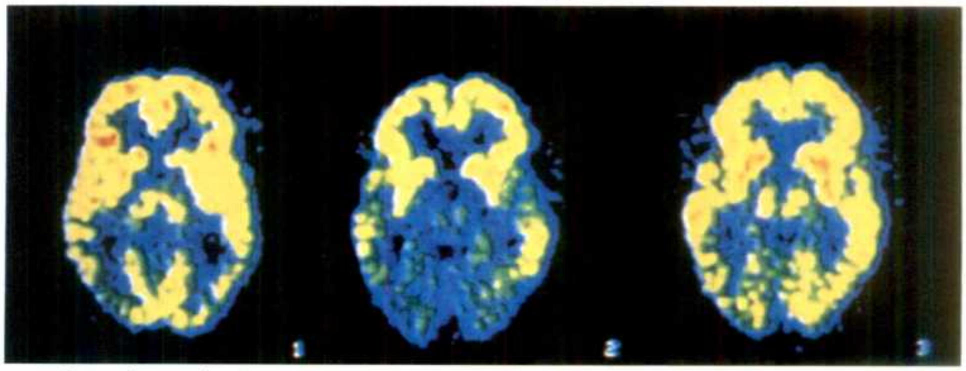


*Extrapolated from data of the Centers for Disease Control: *Morbidity and Mortality Weekly Report.*

Helps patients live better

Improvement following RETROVIR therapy demonstrated by positron emission tomography[3]

PET scans of normal control (1) and patient with HIV-related neuropathy and dementia before (2) and after (3) 13 weeks of RETROVIR therapy



Normal control (1)

Patient before RETROVIR therapy (2)

Patient following RETROVIR therapy (3)

A homogeneous pattern of glucose metabolism can be seen in the normal control (1) as compared with the heterogeneous pattern in the patient prior to RETROVIR therapy (2). After 13 weeks of treatment, the abnormal pattern has partly resolved (3).

Guidelines for successful management

- RETROVIR therapy is generally well tolerated by many patients, particularly those with higher T4 cell counts and more intact bone marrow function at the start of therapy
- Hematologic monitoring is recommended every two weeks; there is a possibility of bone marrow toxicity and subsequent RBC and WBC depression
- Concomitant use of acetaminophen with RETROVIR may potentiate granulocytopenia; other drugs that may interfere with RETROVIR metabolism should be avoided.

LIBRARY

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (ZIDOVUDINE) Capsules

WARNING: THERAPY WITH RETROVIR (ZIDOVUDINE) IS OFTEN ASSOCIATED WITH HEMATOLOGIC TOXICITY INCLUDING GRANULOCYTOPENIA AND SEVERE ANEMIA REQUIRING TRANSFUSIONS (SEE WARNINGS).

IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OI'S) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than 200/mm³ in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss ($\geq$ 15 lbs or $>$ 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 cells/mm³ at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of $<$750/mm³. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count $<$ 1000/mm³ or hemoglobin $<$ 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 μg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/TK$^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 μg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 μg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 μg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 μg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in th placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudin 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicitie was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entr and directly related to dose and duration of therapy. The frequency of granulocytopenia and anemi according to the patients' CD4 (T4) levels is shown in the following table:

	Pretreatment CD4 (T4) Levels			
	≤200/mm³		>200/mm³	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia (< 750/mm³)	47%	10%	10%	3%
Anemia (Hgb < 7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudin examining the degree of change when compared to baseline, as shown in the table below, may be mo informative.

		Pretreatment CD4 (T4) Levels			
		≤200/mm³		>200/mm³	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	>50%	55%	19%	40%	13%
Anemia	>25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasi macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseli symptoms and clinical abnormalities. The following table summarizes those reported clinical adver events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nause insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are list below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudi recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pa lymphadenopathy.

Cardiovascular: vasodilation.

Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gum rectal hemorrhage, mouth ulcer.

Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.

Nervous: anxiety, confusion, depression, emotional lability, nervousness, syncope, loss of men acuity, vertigo.

Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.

Skin: acne, pruritus, urticaria.

Special senses: amblyopia, hearing loss, photophobia.

Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (t 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and durat of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serio anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of $<$7.5 g/dL or reduction of $>$25% of baseline) and significant granulocytopenia (granulocyte count of $<$750/mm³ or reduction of $>$50% from baseline) m require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anem dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occu following dose modification, gradual increases in dose may be appropriate depending on hematolo indices and patient tolerance.

References: **1.** Fischl MA, Richman DD, Grieco MH, et al: The efficacy of azidothymidine (AZT) in t treatment of patients with AIDS and AIDS-related complex: A double-blind, placebo-controlled tr *N Engl J Med* 1987;317:185-191. **2.** Acquired Immunodeficiency Syndrome (AIDS) Weekly Surveilla Report: United States Reported to CDC, July 9-12, 1987. **3**. Yarchoan R, Brouwers P, Spitzer AR, et Response of human-immunodeficiency-virus-associated neurological disease to 3'-azido-3'-deo thymidine. *Lancet* 1987; 1:132-135. **4.** Data on file, Burroughs Wellcome Co.

Burroughs Wellcome Co., Research Triangle Park, North Carolina 277

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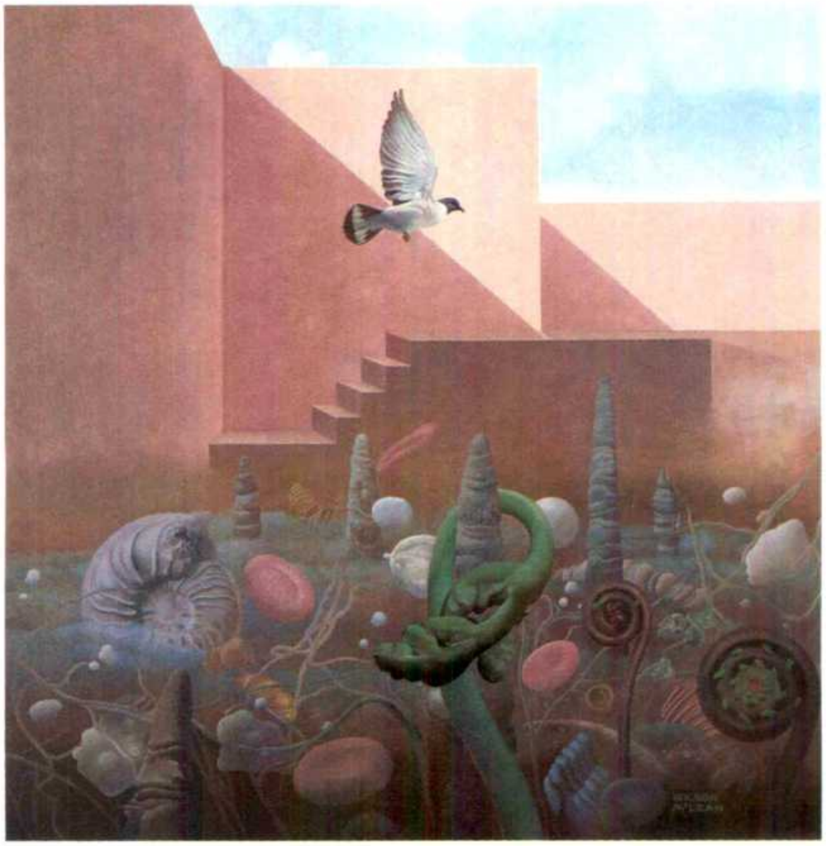

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The Leukotrap System removes 90% of leukocytes and residual red cells with only 10% platelet loss.

Leukotrap™ Pooling Bag shown after completed procedure with leukocytes and residual red cells trapped in the pouch.

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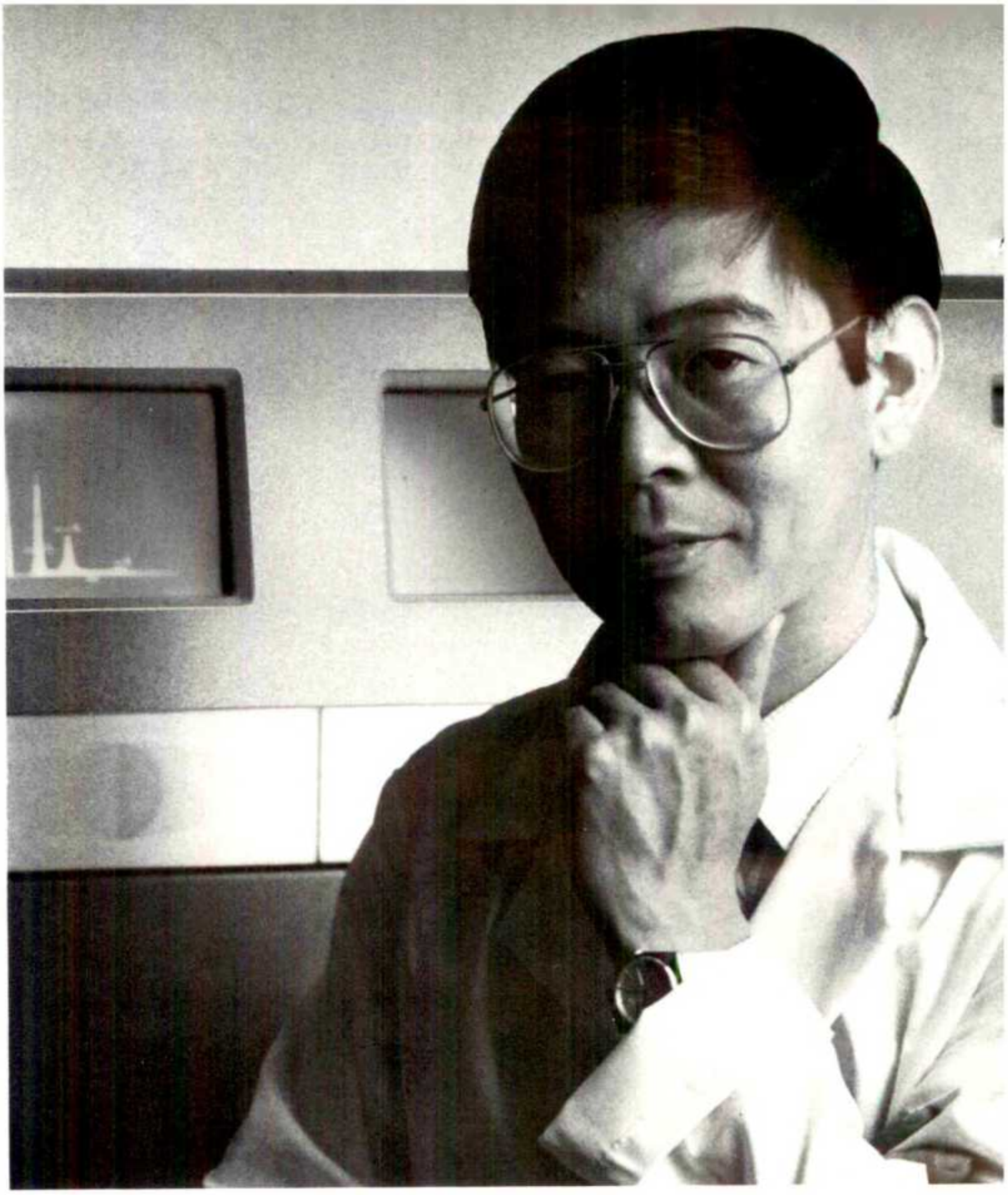
*"For
DNA analysis
of tumor cells, CVs
are very important.
If you don't have good
Vs, you sometimes can't
distinguish between
normal and
malignant cells."*



Peter Keng, Ph.D.
Associate Professor of Radiation Oncology
University of Rochester
School of Medicine and Dentistry

Dr. Keng was interviewed and photographed June 23, 1987, at his laboratory in the Cancer Center at Rochester, New York.

There's no substitute
for experienc

Dr. Peter Keng of the University of Rochester School of Medicine and Dentistry, New York, heads the Cancer Center flow cytometry laboratory where his primary research consists of DNA analysis of tumors. In this recent interview, Dr. Keng discusses the advantages of the EPICS® Profile clinical flow cytometry analyzer from his experience in terms of the particular needs and applications of his laboratory.

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"There's nothing like Profile's convenience for a multi-user facility such as ours where speed and ease of use is so important. With 20 to 30 researchers using one analyzer, set up and execution are very rapid. The software allows one to enter the sample name and protocol as well as analyze the data in a few easy steps. Profile doesn't intimidate people. Once you use Profile, you feel comfortable with it. It really increases productivity."

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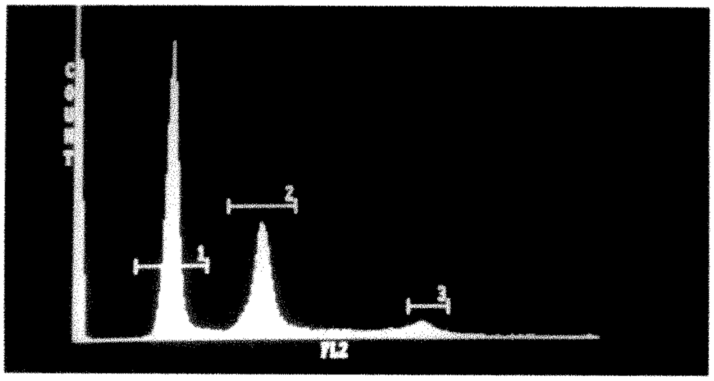

Propidium iodide stained nuclei from a dissociated human hepatoma tumor.
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Region 3: Mitotic cells from malignant population (G_2/M)

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Formation of B-lymphocytes and other blood cells in tissue.

For ITP Patients

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

For patients with acute and chronic idiopathic thrombocytopenic purpura (ITP), maintaining normal platelet levels may be a life-threatening problem. They often need fast, aggressive therapy that will elevate platelet counts rapidly. They need Gamimune® N.

IGIV treatment with Gamimune® N may effect a therapeutic increase in platelets in both children and adults with ITP and, in some cases, allows the possibility of major surgery.[1] In one study,[1] 62.5%(10 of 16) adult patients and 83.3% (5 of 6) pediatric patients demonstrated a substantial response to an initial course of therapy with Gamimune® N.[*][†]

Gamimune® N contains at least 98% gamma globulin and is the only ready-to-use liquid IGIV available. Cutter's patented fractionation method (based on Cohn-Oncley methods 6 and 9) assures Gamimune® N contains no heterologous proteins, with no detectable levels of protein aggregates or vasoactive enzymes.[2] And studies show *in vitro* inactivation of HIV (HTLV-III virus[‡] during production of Gamimune® N.

Gamimune® N. Enhancing the natural process with fast, aggressive therapy for ITP in both children and adults.

---

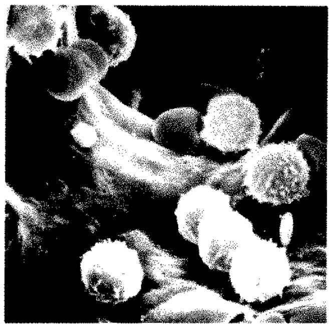
[*] It is not possible to predict which patients with ITP will respond to therapy.

[†] In patients who respond to therapy, a rise in platelet count is generally rapid (within one to five days), transient (most often lasting from several days to several weeks), and should not be considered curative.

[‡] The Cohn-Oncley process used to produce Gamimune® N has been challenged *in vitro* with a virus spike of 1 x 10^5 TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III) and found to remove and/or inactivate the viral challenge. In addition, Gamimune® N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III).[3]

1 Gamimune® N Product License Application
2 Lundblad JL, Mitra G, Sternberg MM, et al. Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(suppl 4): S382-S390
3 Mitra G, Wong MF, Mozen M, et al. Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26:394-397

See adjacent page for brief summary of prescribing information.



GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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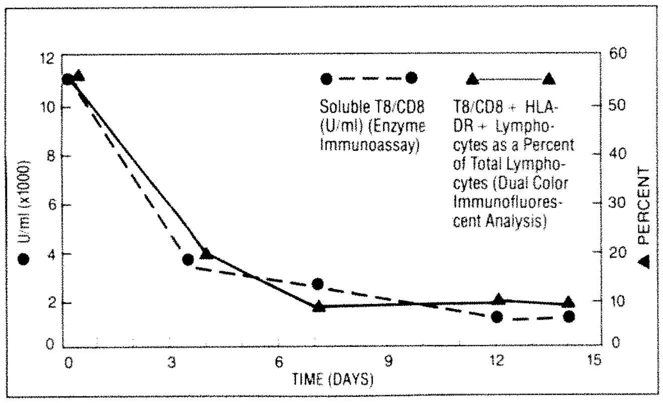

Correlation of soluble CD8 with CD8 + HLA-DR + T Lymphoctyes (Patient E) (3)

A Measure of T8/CD8 + Cell Activation

- In disseminated disease, soluble T8/CD8 has been shown to vary with the number of activated T8/CD8 + cells in peripheral blood as measured by HLA-DR (3).
- Levels of soluble T8/CD8 parallel the decline of T8/CD8 + HLA-DR + cells during the resolution of disease (3).
- Measurement of soluble T8/CD8 may lead to a better understanding of T8/CD8 + suppressor/cytotoxic T cell activity.

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- Reliable, consistent performance with fresh or frozen serum, plasma, or cell culture supernatants.
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AA1011	ACT-T-SET® TLiSA1 Purified Antibody, 100 Tests
AA2011	ACT-T-SET® TLiSA1 FITC Conjugated Antibody, 100 Tests

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1. Fujimoto, J., *et al.*, Spontaneous Release of the Leu-2 (T8) Molecule from Human T Cells, J. Exp. Med., 159: 752-766, 19
2. Pui, C.H., *et al.*, submitted.
3. Tomkinson, B., *et al.*, Released CD8 Levels in Immune Activati in press.

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Radiogenic mucositis or herpes simplex?

Early intervention can prevent or reduce HSV complications

A high percentage of immunocompromised patients are at risk for developing HSV infections. Timely therapy with an antiviral agent can avoid the incidence of secondary complications, such as visceral dissemination, and prolonged hospital stay.

Appearances may be deceiving

Both slides are herpes simplex. In immunocompromised patients, mucositis and stomatitis that appear to result from radiation therapy or immunosuppression may actually be caused by herpes simplex. Differential diagnosis can be problematic, but appropriate laboratory tests can aid in the determination.

There is an effective therapeutic agent available

ZOVIRAX I.V. stops viral replication, speeds healing, and reduces pain. It is effective antiviral therapy for treatment of herpes simplex viral infections.

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(acyclovir sodium)
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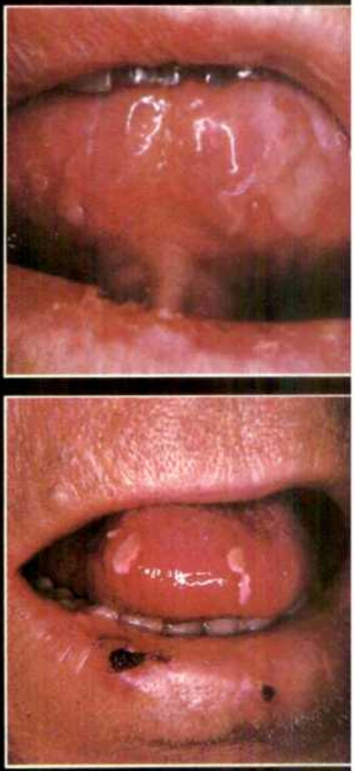
The diagnostic challenge in immunocompromised patients...



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STERILE POWDER / I.V. INFUSION

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency—4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION—RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS***—5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS—The same dose given above—administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

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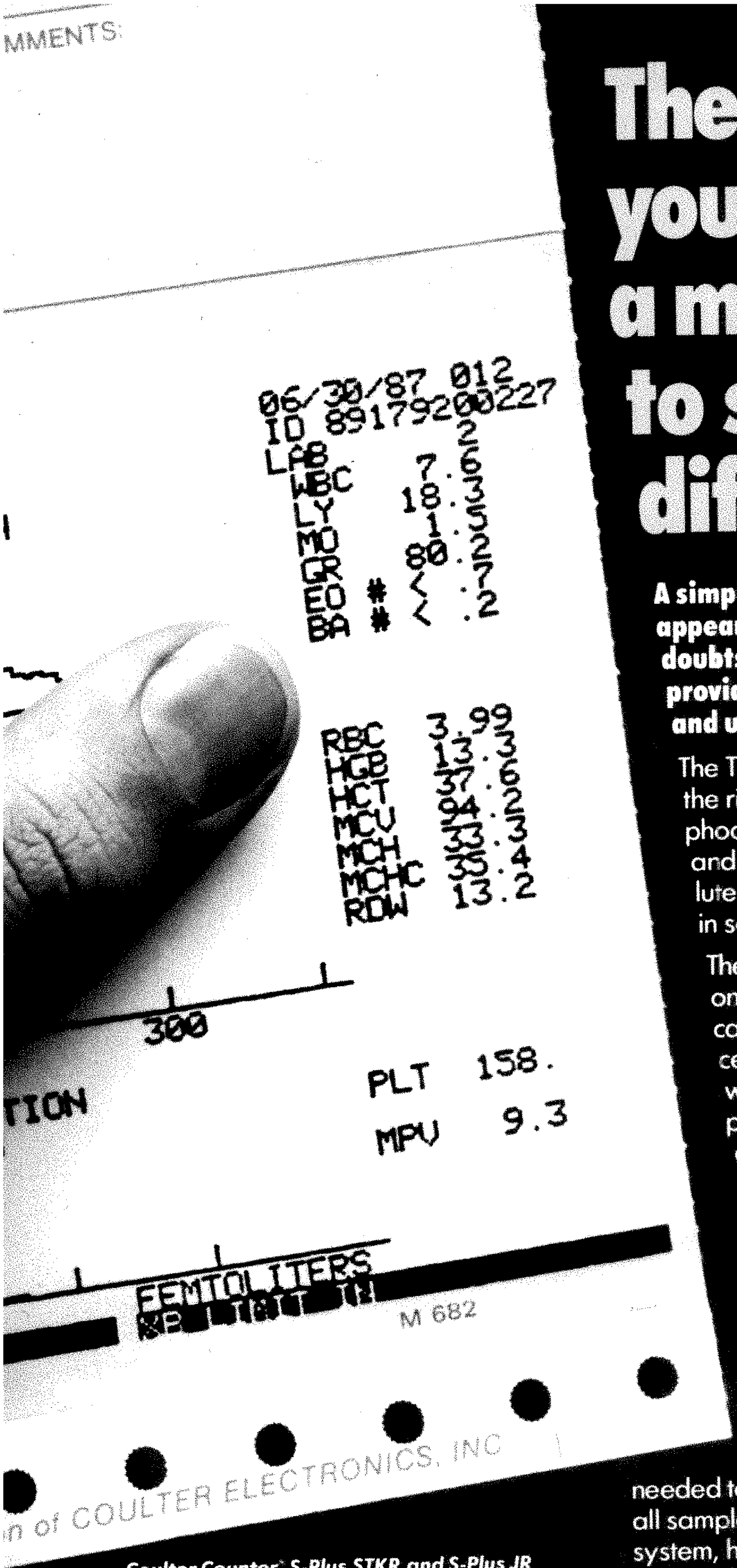

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 WBC     7.6
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  MO     1.5
  GR    80.2
  EO #  < .7
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HCT   37.6
MCV   94.2
MCH   33.3
MCHC  35.4
RDW   13.2

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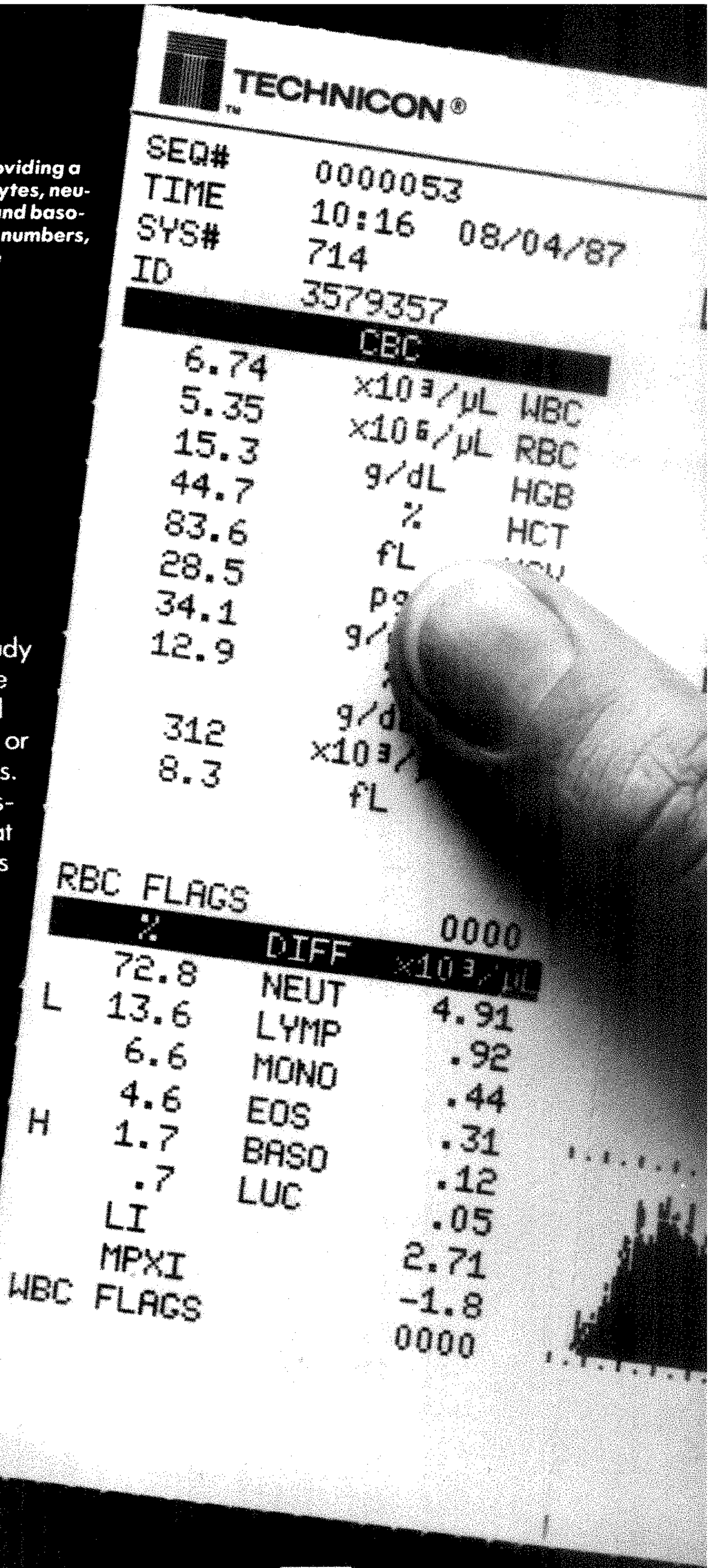

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  15.3    g/dL     HGB
  44.7    %        HCT
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  .7     LUC      .05
  LI              2.71
  MPXI            -1.8
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linician Acceptance of Automated ifferentials

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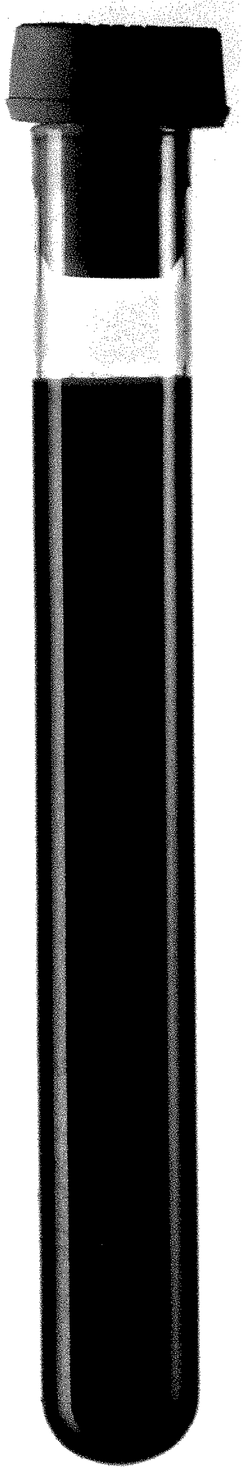

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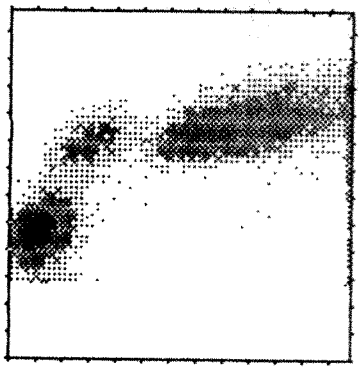

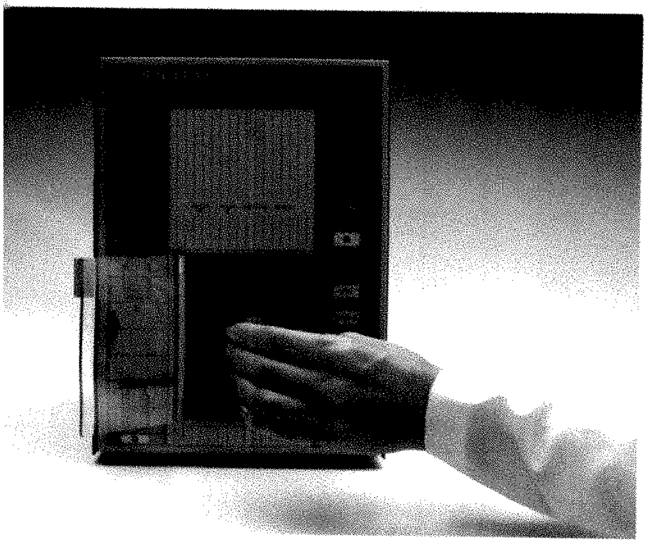

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REVIEW ARTICLE

The 5q− Abnormality

By Stephen D. Nimer and David W. Golde

LOSS OF A PORTION of the long arm of chromosome 5 is seen predominantly in therapy-related myelodysplastic syndromes and acute leukemias, as well as in a unique clinical disorder known as the 5q− syndrome. Recently, many growth factor and growth factor receptor genes have been localized to the long arm of chromosome 5, and several of these genes have been found to be deleted in patients with a 5q− chromosomal abnormality. These findings raise the possibility that alteration of expression of these genes may play a pathogenetic role in these hematologic disorders. In this review, we discuss the clinical and molecular biological findings associated with the 5q− abnormality.

THE 5Q− SYNDROME

Three patients with long-standing idiopathic refractory anemia and an interstitial deletion of the long arm of chromosome 5 were first described by van den Berghe et al in 1974.[1] Two additional patients were reported in 1975,[2] and a new hematologic syndrome was established, the 5q− syndrome. Scores of patients with refractory anemia and a 5q− chromosomal abnormality have since been reported,[3-6] and a distinct clinical entity has emerged. Although deletions of the long arm of chromosome 5 have been seen in a variety of clinical settings,[7,8] patients with the 5q− syndrome share similar morphologic, cytogenetic and clinical features. Precise diagnostic criteria for the 5q− syndrome have not been established. But patients typically present with refractory macrocytic anemia, an interstitial deletion involving the long arm of chromosome 5, and morphologic abnormalities of their megakaryocytes.

The first five patients reported with the 5q− syndrome ranged in age from 32 to 80 years old, and included four women and one man. Three had splenomegaly, and all presented with complaints related to their anemia.[1,2] Subsequent patients have had similar characteristics; ie, there is a marked female predominance, and the median age of the patients is in their mid to late 60s. Splenomegaly is seen in ~25%.[9]

Considerable variability in both bone marrow and peripheral blood findings have been noted in patients with the 5q− refractory anemia syndrome and, depending on the precise bone marrow findings, these patients can be assigned one of the French-American-British (FAB) subclassifications of myelodysplastic syndromes[10] as well. Bone marrow cellularity is usually increased or normal, and there are decreased numbers of erythroid precursors with moderate dyserythropoiesis. Although none of the initial five 5q− patients had ring sideroblasts noted in the marrow, patients with the 5q− syndrome may have increased numbers of ring sideroblasts, and some may be classified as having refractory idiopathic sideroblastic anemia (RISA). Occasional patients have a slight increase in the number of myeloblasts, but WBC maturation is usually normal. Rarely, patients have >5% myeloblasts in the bone marrow, and could be characterized as having a refractory anemia with excess blasts (RAEB), whereas patients without ring sideroblasts or excess myeloblasts would be classified as having refractory anemia (RA). The most consistent bone marrow finding in these patients is the presence of abnormal megakaryocytes containing small nonlobulated nuclei. Nearly all patients have abnormal megakaryocyte morphology, and most have an increased number of megakaryocytes as well.

Blood smears reflect the abnormalities seen in the bone marrow. Aniso- and poikilocytosis of the RBCs is found with many macro-ovalocytes. WBC morphology is generally normal, but marked platelet anisocytosis is present, with many giant platelets.

Electron microscopy was performed on bone marrow and blood from three of the original five patients.[2] RBCs were frequently adherent to one another, and marrow erythroblasts were seen in clusters, with intimate membrane contact between cells. Forty percent of neutrophils had an abnormally high number of primary granules, whereas specific granules were rare in both mature neutrophils and differentiated neutrophil precursors. Megakaryocytes were generally small, with nonlobulated round or oval nuclei, and liberation of platelets often appeared abnormal. Giant platelets were

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frequently observed, and although specific granules were present, dense granules were frequently absent.

Few functional studies have been reported on mature blood cells from patients with the 5q− syndrome. Because of the consistent platelet and megakaryocyte abnormalities, platelet function was assessed in four of the five original patients. These patients had normal bleeding times in the presence of normal or elevated platelet counts, but platelets from three of the four patients demonstrated decreased adhesion to glass beads, and all demonstrated decreased platelet aggregation in response to collagen and epinephrine.[2]

In vitro culture of bone marrow from these patients has demonstrated normal numbers of CFU-GM,[3,11] but markedly reduced to absent BFU-E colonies.[11] This pattern seems to reflect the predominant peripheral blood abnormalities seen in the patients, namely that of refractory anemia.

Much interest developed in the 5q− syndrome because it represented a defined hematologic syndrome associated with a specific chromosomal abnormality. Cytogenetic investigations performed on the first five patients with the 5q− syndrome revealed the consistent deletion of approximately two-thirds of the long arm of chromosome 5, without apparent translocation of the deleted segment, in marrow cells cultured without PHA for 24 to 48 hours. The deletion was thought to be interstitial, possibly involving bands 5q12-5, 5q21-3. Additional data from other patients generally shows a proximal breakpoint in the region 5q13-q15, and a distal breakpoint in region 5q31-33,[12] although occasional patients with other breakpoints have been reported (a proximal breakpoint at q23, a distal breakpoint at q23), and at least sixteen patients have been described with a del(5)(q31q35).[13,14] No cytogenetic abnormalities have been reported in skin fibroblasts or PHA-stimulated peripheral blood mononuclear cells from patients with the 5q− syndrome, reflecting the acquired nature of the disorder and the fact that most circulating T lymphocytes are not part of the abnormal clone.

Although some variability occurs in the breakpoints of the 5q− chromosomes among different patients, several investigators have noted that the deleted long arm segments consistently occurred in the region of the band 5q22-q31, and postulated a localized genetic change that could contribute to the development or progression of either abnormal or frankly malignant cell growth.[4,15] Recently, various hematopoietic growth factor and growth factor receptor genes have been localized to the long arm of chromosome 5[16-20] in a region frequently deleted in patients with a 5q− abnormality. These findings have led to speculation regarding a pathogenetic role for these genes in the resulting clinical syndromes (vide infra).

None of the first five reported patients developed acute leukemia within the observation period of the study (up to 6 years),[2] but subsequent reports from Rowley and van den Berghe raised questions about the natural history of patients with this chromosomal abnormality.[21,22] Both reports described patients with 5q− chromosomal abnormalities and hematologic disorders that terminated in acute leukemia. Of the five patients with acute leukemia reported by van den Berghe,[21] three were elderly women, including one with a prior history of refractory anemia. The other two patients were a 59-year-old man with subacute myelogenous leukemia, and a thirteen-month-old boy with acute myelogenous leukemia (AML) and a short prodromal period. Rowley reported on five female patients with a 5q− abnormality who developed acute leukemia.[22] These patients all had different clinical histories, including one patient with agnogenic myeloid metaplasia, and one with multiple myeloma. Thus, these patients did not share a clinical syndrome, but rather a common chromosomal abnormality. In addition to deletion of the long arm of chromosome 5, all ten patients had additional chromosomal abnormalities that occurred at an unknown time in their clinical course. Patients with the classical 5q− syndrome have the 5q− chromosomal anomaly as an isolated abnormality[5,8,13]; thus the ten patients who developed leukemia were both clinically and karyotypically different than patients with the "5q− syndrome." Conclusions about the natural history of the 5q− syndrome cannot easily be made from patients with variant clinical and cytogenetic findings. The presence of a 5q− abnormality in a patient with acute leukemia does not establish the existence of an antecedent 5q− syndrome.

Patients with the characteristic 5q− syndrome have been reported to develop acute leukemia,[23,24] but the presence of the 5q− chromosomal deletion by itself, in a patient with a refractory anemia, does not appear to be associated with a high likelihood of subsequent acute leukemia (possibly 5% to 10%). In a 1983 review of 24 patients with an isolated 5q− chromosomal abnormality and refractory anemia, only three developed acute leukemia.[8] Patients with a 5q− deletion who either have additional chromosomal abnormalities at presentation, or who evolve chromosomal abnormalities in addition to an isolated 5q− deletion, appear to have a higher likelihood of evolution to acute leukemia than do patients with an isolated 5q− abnormality,[5,8] but a definitive statistical analysis proving this has not been done.

Management of patients with the 5q− syndrome is supportive. Since these patients do not generally manifest leukopenia, they are not unduly susceptible to infections. Their platelet counts are normal or elevated, and despite the reported abnormal in vitro platelet function tests, they do not experience abnormal bleeding nor unusual thrombotic episodes. Thus, the major manifestation of this syndrome relates to the refractory anemia and the need for RBC transfusions. The severity of the refractory anemia varies markedly from patient to patient. Some patients have not required transfusions during several years of follow-up, whereas others require frequent and regular transfusions to prevent the symptoms of anemia. Patients receiving repeated red cell transfusions are at risk for the infectious complications of blood transfusions, as well as iron overload. Subcutaneous administration of desferoxamine, an iron chelating agent, should be instituted in those patients requiring frequent transfusions.

Syngeneic bone marrow transplantation was performed for a 29-year-old man with a 5q− refractory anemia and iron overload, and was successful in eradicating the 5q− clone and allowing successful treatment of his iron overload.[25]

5Q– AND DE NOVO AML

Adult AML is associated with chromosomal abnormalities in 50% to ≥90% of the cases.[26] Approximately half of these patients have a mixture of normal and abnormal metaphases, whereas half have only abnormal metaphases. In one large survey, 85 of 496 patients with de novo AML had abnormalities of chromosome 5 and, in approximately half of these patients (47%), this abnormality was a 5q– chromosome.[15] Unlike the situation of 5q– refractory anemia where the 5q– is usually an isolated abnormality, most patients with de novo AML and a 5q– have additional chromosomal abnormalities, such as trisomy 8, 11, or 21, or monosomy 7 or 17.[9]

The Fourth International Workshop on Chromosomes and Acute Leukemia reported 660 patients with de novo AML and 50 patients (7.6%) had abnormalities of chromosome 5 (monosomy 5 or 5q–), with or without abnormalities of chromosome 7. The 29 patients with isolated abnormalities of chromosome 5 had a poor prognosis; only 28% achieved complete remission after aggressive induction chemotherapy. The median duration of remission was 4 months, and the overall median survival of these patients was 4 months, significantly worse than patients with other chromosomal abnormalities or those without chromosomal abnormalities.[27]

In several studies, patients with a history of occupational exposure to potential carcinogens have been shown to have a higher incidence of chromosomal abnormalities in their leukemic cells than those without such a history. An association between abnormalities of chromosomes 5 and 7 in patients with de novo ANLL and a significant history of occupational exposure was first noted by Mitelman et al,[28] and follow-up studies (Brandt et al in Lund, Sweden[29] and Golomb et al at the University of Chicago)[30] have confirmed this association. Combining their studies, 68 of 236 patients with de novo AML gave a history of significant exposure to chemicals, petroleum solvents, pesticides, or industrial metals. Abnormal karyotypes were noted in 75% of the exposed patients (51 of 68), compared with 36% in the nonexposed patients (60 of 168) ($P < .05$). Abnormalities of chromosomes 5 and 7 were seen in 37% of the exposed patients, compared with 12% in the nonexposed patients, again, a significant difference. In these studies, these specific chromosomal abnormalities were not prognostically different than other chromosomal abnormalities occurring in patients with de novo acute leukemia, which contrasts to the findings of the Fourth International Workshop on Chromosomes and Acute Leukemia.[27]

CHROMOSOME 5 ABNORMALITIES AND THERAPY-RELATED AML AND MYELODYSPLASTIC SYNDROMES

The occurrence of acute myeloid leukemia in patients who have been treated with chemotherapy and/or radiotherapy for other malignant diseases has been increasing, probably reflecting our improved success at prolonging survival with these therapies. Patients with Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, and ovarian carcinoma, as well as other malignancies, are at increased risk for the development of acute leukemia.[31-33] As reported by Rowley et al in 1981 and recently updated in 1986, patients with therapy-related acute leukemia or therapy-related myelodysplastic syndrome commonly have a clonal abnormality of chromosome 5 and/or 7 consisting of loss of all or part of the long arm of the chromosome.[34,35] Fifty-five of the 63 patients studied had such an abnormality (87%), and 31 of those 55 patients had either loss of chromosome 5, or the 5q–. In contrast, only ~16% of patients with de novo AML have loss of the long arm of chromosome 5 or 7. The difference between de novo acute leukemia and therapy-related acute leukemia was further supported by the observation that only one of 63 patients with therapy-related acute leukemia had a t(15;17) or t(8;21) translocation, two translocations commonly seen in patients with de novo acute leukemia.

In this study, 14 patients lost one copy of chromosome 5, whereas 17 patients had an interstitial deletion of the long arm of chromosome 5 (5q–). No association of this chromosomal abnormality or any other cytogenetic abnormality was seen with regard to the sex or age of the patients, in contrast to the predominantly elderly female population of patients with 5q– syndrome. No significant association was seen in this study between the type of primary therapy (ie, chemotherapy, radiotherapy, or combined modality therapy), and the occurrence of the 5q– abnormality. Survival of all patients was poor, with no correlation between median survival and the presence of any particular chromosomal abnormality.

Abnormalities of chromosome 5 were more frequent in patients with therapy-related myelodysplastic syndrome than in those with therapy-related AML and no preleukemic phase. The presence of a 5q– abnormality in a patient with therapy-related myelodysplastic syndrome, however, was not helpful in predicting whether the patient would evolve therapy-related AML.

Some have suggested that abnormalities of chromosomes 5 and/or 7 may be related to the form or intensity of therapy given for the primary disease.[36,37] For example, Arthur and Bloomfield found, by combining their study of 20 patients with 66 previously published cases, a significantly higher incidence of abnormalities of chromosomes 5 and/or 7 in patients who received combination chemotherapy (≥3 agents) or combined radiotherapy and chemotherapy, compared with patients who received radiotherapy alone, or single-agent chemotherapy alone.[37] Pederson-Bjeergard et al[38] suggested that characteristic karyotypic abnormalities could be identified in patients with therapy-related leukemia if one looked at those who had had a preleukemia phase, received chemotherapy, and became leukemic 30 to 60 months after the start of therapy. They suggested that karyotypic abnormalities occurring in other settings, such as after radiation therapy alone or after <24 months or >60 months after the start of primary therapy, occurred with no greater frequency than those occurring in patients with *de novo* acute leukemia. They, as well as others, have noted the more frequent occurrence of abnormalities of chromosomes 5 and/or 7 in patients receiving combination chemotherapy or combined modality treatment, but it appears as though these karyotypic abnormalities can be seen in therapy-related myelodysplastic syndromes or therapy-related acute leukemia, regardless of the type or intensity of primary therapy. As a rule, patients with therapy-related leukemia do quite poorly; therefore, the presence of a 5q− abnormality cannot be shown to be of prognostic significance in this setting.

THE 5q- ABNORMALITY IN OTHER DISORDERS

In addition to its occurrence in patients with the 5q- syndrome or AML, the 5q-deletion has also been reported in patients with a variety of myeloproliferative disorders. The 5q- abnormality has been found in patients with polycythemia vera,[8] and occasionally in patients with chronic myelogenous leukemia,[39] essential thrombocythemia,[7] and myeloid metaplasia.[40] It has also been reported in patients with pure red cell aplasia,[41] acute lymphocytic leukemia,[42] and rarely other lymphoproliferative disorders.

THE 5q- CHROMOSOME

Certain hematologic malignancies such as Burkitt's lymphoma and chronic myelogenous leukemia have been associated with specific chromosomal abnormalities: an 8;14 translocation in Burkitt's lymphoma, and the 9;22 translocation (Philadelphia chromosome) in CML.[43,44] Molecular biologic analyses have shown that these chromosomal abnormalities result in the translocation of known proto-oncogenes from one chromosome to another, which can result in either altered regulation of the proto-oncogene (as is the case for c-*myc* in Burkitt's lymphoma) or in the production of an altered gene product (as is the case for the p210-*bcr*-c-*abl* fusion product in CML).[45,46] It had long been postulated that abnormal chromosome patterns could be intimately associated with the malignant phenotype of the tumor cell, and now it is possible to link characteristic chromosomal abnormalities with specific alterations in the genes localized to the involved regions.

The association of an interstitial deletion of the long arm of chromosome 5 with a specific hematologic syndrome has prompted an investigation into what genes are located on the long arm of chromosome 5, and what role they may play in the resultant clinical syndrome.

As seen in Table 1, a variety of hematopoietic growth factor and growth factor receptor genes have been localized to the long arm of chromosome 5. Among these genes are the genes encoding three of the four myeloid colony-stimulating factors, as well as the genes encoding the PDGF receptor,[47] the β2 adrenergic receptor,[49] endothelial cell growth factor,[50] the glucocorticoid receptor,[51] dihydrofolate reductase,[52] and other genes as well (Table 1). Colony-stimulating factors were initially identified, based on their ability to promote the growth and maturation of murine myeloid progenitor cells in semisolid media culture. Four distinct murine CSFs were isolated and purified, and found to stimulate the proliferation of hematopoietic progenitor cells and enhance the functional capabilities of mature myeloid effector cells (reviewed in reference 53). These were named according to the predominant types of colonies that result when the factor is added to normal bone marrow progenitor cells: macrophage colony-stimulating factor (M-CSF), which acts on cells of the monocyte-macrophage lineage; granulocyte-macrophage colony-stimulating factor (GM-CSF), which acts on granulocytes (including eosinophils) and macrophages; granulocyte colony-stimulating factor (G-CSF), which acts on granulocytes; and multi CSF, or interleukin 3 (IL-3), which acts on erythroid, megakaryocyte, and a variety of myeloid progenitors. The four human counterparts of these myeloid CSFs have now been identified and cloned. The genes encoding three of these four hematopoietic growth factors have been localized to the 5q region, whereas G-CSF has recently been shown to be on 17q.[20]

Table 1. Genes Localized to the Long Arm of Chromosome 5

GM-CSF	5q21-32,[16] 5q23-31[17]
IL-3	5q23-31[19]
PDGF receptor	5q31-32[43]
β2-adrenergic receptor	5q31-32[44]
c-*fms*	5q33.2-33.3[17,20]
M-CSF	5q33.1[20]
ECGF	5q31.3-33.2[45]
Dihydrofolate reductase	5q11-22[47]
Glucocorticoid receptor	5q11-13[46]

The diphtheria toxin sensitivity, arylsulfatase B, chromate resistance and emetine resistance genes have also been localized to 5q.[48]

The first hematopoietic growth factor gene localized to the long arm of chromosome 5 was the gene encoding GM-CSF, which is ~2.5 kb in length, and contains four exons.[16] Human GM-CSF is expressed in activated but not resting T lymphocytes, and in HTLV-infected T lymphoblast cell lines.[54] Tumor necrosis factor or IL-1–stimulated fibroblasts and endothelial cells can also express GM-CSF,[55,56] as can stimulated peritoneal macrophages.[57]

Human GM-CSF is a 22,000 dalton (d) glycoprotein that stimulates the formation of pure neutrophil, pure eosinophil, pure monocyte, and mixed neutrophil/monocyte colonies from normal human bone marrow.[58] Activity on erythroid and megakaryocyte colony growth can also be documented under certain circumstances.[69-61] GM-CSF stimulates colony formation by the KG-1 and HL-60 leukemia cell lines,[58] and can enhance the function of mature neutrophils, eosinophils, and monocytes. In addition to these in vitro activities, in vivo activity of GM-CSF has now been demonstrated in both monkeys and man.[62,63] Using somatic cell hybrid analysis and in situ hybridization, the GM-CSF gene has been localized to the human chromosome region 5q21-q32.[16] A second group, also using in situ hybridization, localized the GM-CSF gene to 5q23-31, and reported three patients with 5q-interstitial deletions (two with refractory anemia, and one with acute myelogenous leukemia) who had deletion of one of their GM-CSF alleles.[17]

The gene encoding IL-3, or multi-CSF, contains five relatively small exons,[64] and the murine IL-3 gene has been located within ~230 kb of the murine GM-CSF gene on chromosome 11.[65] There is evidence that the human IL-3 gene, which has been localized to region 5q23-31, is located within 10 kb of the human GM-CSF gene.[66] IL-3 has effects on myeloid, erythroid, and megakaryocyte growth, presumably acting at an earlier stage in stem cell differentiation than does GM-CSF. The biological activities of human IL-3 are currently being examined, but enhancement of human eosinophil function by recombinant gibbon IL-3 has been demonstrated.[67] Thus far, production of IL-3 has been observed only by activated T lymphocytes, implying tight regulation of expression of the IL-3 gene.

M-CSF, or CSF-1, is quite different from the other three CSF molecules. It is a homodimer with a mol wt of ~45,000, whereas the other CSFs are ~20,000 d monomers. GM-CSF and G-CSF are not detectable in serum or urine, but M-CSF is readily detected.[68] M-CSF is encoded by a single gene consisting of many exons that are distributed over 18 to 22 kb. The M-CSF gene has been localized to 5q33.1.[20] Unlike IL-3 and GM-CSF, M-CSF is produced by many tissues and cells, including fibroblasts, endothelial cells, stimulated monocytes, and a variety of malignant cell lines, such as the Mia Paca pancreatic cancer cell line.[69] M-CSF stimulates bone marrow progenitors to become mononuclear phagocytes, and also promotes the proliferation of mature macrophages.[69,70]

It is interesting to note that the M-CSF gene and the c-fms proto-oncogene are both found on the long arm of chromosome 5, at 5q33-34 by in situ hybridization.[17,20] The c-fms gene product appears to be identical to the receptor for M-CSF.[71] Thus, both the hormone and its receptor are located closely together on chromosome 5. Expression of c-fms has been detected in human bone marrow cells and in peripheral blood mononuclear cells. c-fms expression is also seen in HL-60 cells differentiated towards macrophages (by TPA), but not in HL-60 cells differentiated towards neutrophils (by DMSO).[18] Time course studies using HL-60 cells suggest that expression of c-fms mRNA correlates temporally with the development of the differentiated monocyte-macrophage phenotype.[18]

The CSF-1 receptor has been found on monocytes and macrophages from many sources, including bone marrow, spleen, blood, peritoneal fluid, and the lung.[72] It mediates the effects of CSF-1 or M-CSF, a glycoprotein hormone that acts exclusively on cells of the monocyte-macrophage lineage. M-CSF affects the proliferation and maturation of monocyte-macrophage progenitors, and can stimulate the production of monokines, including IL-1.[70] These monokines can stimulate production of GM-CSF by fibroblast and endothelial cells, and can enhance the activities of a variety of growth factors.[56,73,74]

POSSIBLE LINK BETWEEN GROWTH FACTOR GENES AND CLINICAL SYNDROMES

The association of a specific interstitial deletion of the long arm of chromosome 5 with either malignant or disordered hematopoiesis raises the possibility that the deletion of the involved segment is critical to the process of transformation and clonal growth.[16,17] Several hypotheses have been offered to address the relationship between a specific interstitial deletion and abnormal gene expression.[17] One hypothesis suggests that as a result of this interstitial deletion, juxtaposition of two critical gene sequences occurs, leading to the process of abnormal hematopoiesis. This does not appear to be the case for the 5q-abnormality. There is marked variability in both the proximal and distal breakpoints in these interstitial deletions and, although there are several general classes of breakpoints, they could not result in the consistent juxtaposition of critical genetic elements. Thus, this mechanism is not likely to be involved in the process of transformation. Another hypothesis holds that an interstitial deletion within a chromosome affects genes critically involved in sustaining normal hematopoiesis. At the moment, the status of the GM-CSF, M-CSF, and c-fms genes has been explored in patients with 5q− abnormality, and in all instances, only a single allele has been found to be deleted.[17-20] Several potential mechanisms have been proposed to explain how deletion of one of two alleles of a hematopoietic growth factor gene could result in altered growth of hematopoietic cells.[17] One involves a dose effect, where cells with only a single copy of a gene make an inadequate amount of the gene product to allow for normal cell growth. In another scenario, loss of the wild-type gene results in the unmasking of a recessive mutant allele in a fashion similar to that observed for retinoblastoma and Wilms' tumor.

There are several obstacles in the way of firmly linking abnormalities of growth factor gene expression to the clinical syndromes associated with deletions of the long arm of chromosome 5. The first is that we are really not sure how these factors control the day-to-day production of blood cells, nor what signals regulate their synthesis. The second is that we do not know which cells are responsible for producing the growth factors needed to sustain the ongoing process of hematopoiesis. It is likely that multiple factors and a variety of cells are involved in the regulation of hematopoiesis, including macrophages, T lymphocytes, endothelial cells, and various stromal cells. Disruptions in these interactions could result in disordered hematopoiesis.

An additional difficulty in ascribing any particular gene abnormality to a resulting clinical syndrome is that, with the exception of c-fms, expression of these genes occurs in cells that do not have the 5q− chromosomal abnormality. Griffin et al reported that GM-CSF mRNA can be detected in as many as 50% of leukemia cells cultured in vitro from patients with AML,[75] but it is possible that some in vitro selection of GM-CSF–producing leukemia cells is occurring. If in vivo expression of GM-CSF by myeloblasts does occur, it would more likely be the result of disordered regulation of GM-CSF expression than the result of deletion of a single GM-CSF allele.

Of the genes localized to the long arm of chromosome 5, those encoding M-CSF and the M-CSF receptor (c-fms) could be implicated most plausibly in the variety of clinical findings seen in patients with 5q− abnormality. Simple deletion of a single GM-CSF allele or a single IL-3 allele would be unlikely to result in the abnormalities of myeloid or erythroid cell growth seen in either the 5q− syndrome or therapy-related AML. Expression of IL-3 has been demonstrated only in activated T lymphocytes, whereas the 5q− deletion has not been observed in T cells from patients with the 5q− syndrome. Similarly, GM-CSF expression has been observed only in activated T lymphocytes, and in stimulated fibroblasts, endothelial cells, and macrophages. Given that the 5q− anomaly is an acquired abnormality of myeloid cells, one would not expect fibroblasts (which do not contain a 5q− deletion) or endothelial cells to have abnormal GM-CSF expression, although macrophages with a 5q− deletion could have abnormal GM-CSF expression.

Deletion of c-fms in patients with a 5q− deletion has been shown by several groups.[17,18] All patients have had one copy of the c-fms gene present, although its functional capability is not known. If the 5q− deletion unmasked an abnormal c-fms allele or reduced the number of M-CSF receptors below some critical level, the ability of monocytes to respond appropriately to M-CSF may be impaired. This could result in either cell growth in the absence of growth factors (a step towards transformation), or an inability of the cells to respond appropriately to their growth factors. Differences in the precise molecular defect associated with the 5q− abnormality could determine which condition occurs. It is also possible that M-CSF production is abnormal in patients with the 5q− deletion, and the combined abnormalities result in the monocyte's inability to normally control hematopoiesis. Arguing against many of these speculations is the fact that granulopoiesis is generally normal in the 5q− syndrome, although it is obviously abnormal in AML.

The process of leukemogenesis is likely a multi-step one, and clearly requires more than autonomous growth factor production. Alterations must occur that transform the cell into an invasive cell unable to mature or regulate its growth. As studies looking at the status of these growth factor and growth factor receptor genes in specific clinical situations continue to be reported, it may become possible to implicate abnormalities of specific genes with specific clinical events.

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Molecular Analysis of Burkitt's Leukemia in Two Hemophilic Brothers With AIDS

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In two hemophilic brothers infected by the human immunodeficiency virus (HIV), Burkitt's leukemia developed within 1 year. Both patients were treated by aggressive chemotherapy, and both are still in complete remission for 23 and 14 months, respectively. Sera from both brothers contained anti-HIV antibodies. However, DNA extracted from the tumor cells, when analyzed by Southern blot using a cloned HIV probe, did not reveal HIV-related sequences. Hybridization experiments with an Epstein-Barr virus (EBV) probe revealed the presence of EBV-specific sequences in the tumors' DNA. In both patients' tumors rearranged *c-myc* genes were found. The rearrangements occurred in both genes 3′ to the third exon of *c-myc*, thereby suggesting that a variant chromosomal translocation took place in both cases. Indeed, karyotype analysis of the malignant cells of one of the patients revealed the variant t(2:8) translocation. In contrast to the majority of Burkitt's tumors carrying this translocation, which are κ light-chain producers, cells of our patient expressed λ chains. Furthermore, in both cases the lymphoblasts carried IgG on the surface, again an unusual finding in Burkitt's tumors. Finally, because both patients had an identical HLA phenotype, the role of genetic factors in the development of such tumors should be considered.

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MALIGNANCIES LIKE KAPOSI'S sarcoma or lymphoma are known manifestations of the acquired immunodeficiency syndrome (AIDS).[1-7] The B cell lymphomas in these patients are frequently high-grade B cell malignancies, involve extra nodal sites, and run an aggressive course. Most cases of B cell tumors have been reported in homosexuals and drug addicts, whereas only a few have been described among hemophilic patients with AIDS.[7-11]

Hereby, we report two hemophilic brothers with AIDS who developed Burkitt's leukemia within 1 year. The role of viral infections and genetic factors in the evolution of lymphomas in such patients will be discussed.

PATIENTS AND METHODS

Patients. Patient no. 1 is a 16-year-old boy with severe hemophilia A who had been treated for years with factor VIII concentrates. In March 1983 he presented with a rapidly disseminating cutaneous and visceral varicella-zoster virus (VZV) infection. He had a 1-year history of weight loss, cervical lymphadenopathy, splenomegaly, and oral candidiasis. In January 1985 the lymphadenopathy progressed and became generalized. Anti–human immunodeficiency virus (HIV) antibodies were first assayed at that time and found to be present. A cervical lymph node biopsy specimen taken on March 1985 was diagnosed as diffuse small noncleaved (Burkitt's) lymphoma. A bone marrow aspirate revealed infiltration by lymphoblasts with an L3 morphology that amounted to 30% of the cells. Treatment was begun according to a modified National Institute of Health protocol 7704. After 2 months of receiving chemotherapy, a complete remission was achieved, and treatment was stopped in December 1985. He has remained since then in good general condition except for recurrent infections.

Patient no. 2 is a 21-year-old hemophiliac and the elder brother of patient 1 who had also been treated for many years with factor VIII concentrates. In January 1984, he started to suffer from recurrent upper respiratory infections. In April 1985, anti-HIV antibodies were first found. Concomitantly, a persistent lymphopenia (0.5×10^9/L) and thrombocytopenia (35 to 85×10^9/L) were observed. In December 1985, he presented with severe skeletal pain, and bone marrow biopsy material and aspirates showed heavy infiltration by L3 lymphoblasts that amounted to 80% of the cells. Computed tomography of the abdomen did not reveal any masses. The patient was treated by a modified German Multicenter Trial (BFM) protocol for B cell leukemia, and 1 month later he was in complete remission. Treatment was stopped after 4 months, and since April 1986 he remains in complete remission. An informed consent for bone marrow aspiration and chemotherapy administration was obtained from both patients and their parents.

Methods. Antibodies to HIV were assayed by an enzyme-linked immunosorbent method and confirmed by Western blot with a commercial kit (Du-Pont, Wilmington, DE) according to the manufacturer's instructions.[12,13] Antibodies to Epstein-Barr virus (EBV) early antigen (EA) and viral capsid antigen (VCA) were detected by an indirect immunofluorescence test.[14] Immunophenotyping of the tumor cells, HLA typing, and karyotype analysis were performed as described.[15,16] DNA was extracted from the tumor cells,[17] digested with the appropriate restriction enzymes, separated according to size by electrophoresis in 0.7% agarose, and analyzed by Southern blot hybridization with the relevant radiolabeled probe.[18,19] The *c-myc* probe was the 1.4-kilobase (kb) *Cla*I-*Eco*RI insert of the plasmid pMC41-3RC containing exon 3 of *c-myc*.[20] The EBV probe contains the 4.8-kb *Bam*HI K fragment of EBV, which was cloned in pBR322 and encodes for the first EBV-associated nuclear antigen.[21] The cytomegalovirus (CMV) probe pCM5018 contains the *Eco*RI J fragment that encodes for the 1.9-kb major immediate early RNA.[22] The VZV probe contains the *Eco*RI A fragment.[23] The herpes simplex virus (HSV1) probe (pSG28) contains the *Eco*RI E to K fragments cloned in pBR325.[24] The probe for HIV was the pPol (Du-Pont), derived from the human T-lymphotropic virus III clone λBH10 and contains the 4.9-kb *Bgl*II-*Bgl*II fragment.[25]

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RESULTS

Antibody assays. Serum samples from both brothers were positive for anti-HIV antibodies. Figure 1 depicts the Western blots confirming the serological tests. Both sera showed strong reaction with several HIV proteins, but differences in the pattern of reactivity could be demonstrated: the serum of patient no. 1 did not react against the gag-gene product p17 or against p51.[26,27] The results of determination of the titers of antibodies to EBV-EA and -VCA are summarized in Table 1. The antibody response is consistent with reactivation of EBV infection, as can be found in many AIDS patients.

Both patients had antibodies in high titers to CMV, VZV, and HSV.

Immunophenotype analysis. Bone marrow lymphoblasts from patient no. 1 were HLA-DR+, CALLA$-$, My906$-$, WT1$-$, OKT3$-$, OKT4$-$, OKT6$-$, OKT8$-$, OKT11$-$, Tac$-$, $\gamma+$, $\mu-$, $\delta-$, $\kappa+$ and $\lambda-$.

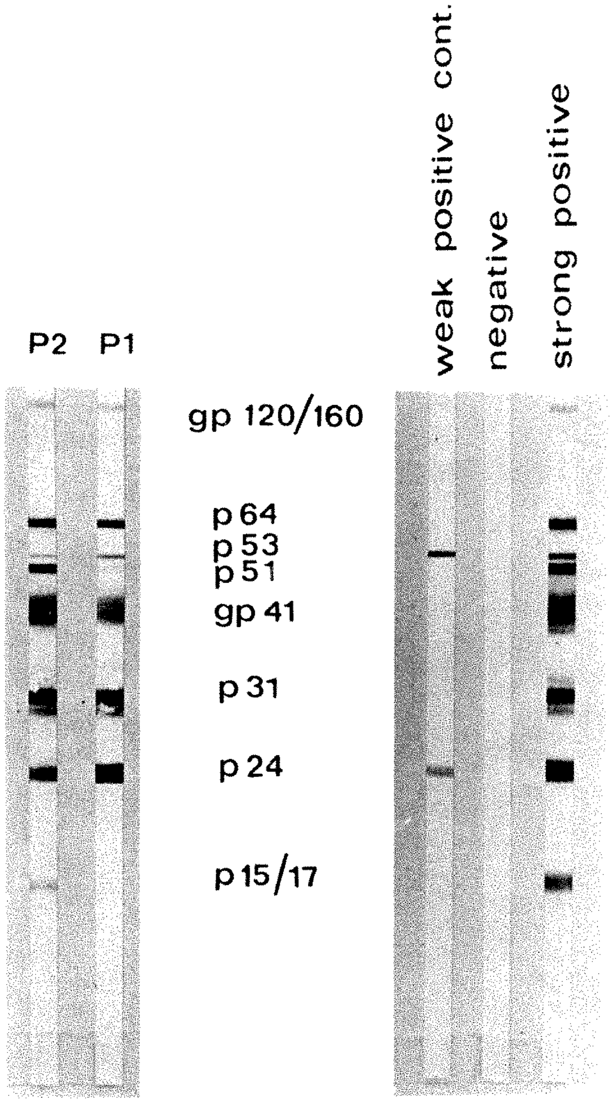

Fig 1. Western blot analysis of sera obtained from both patients. Control samples are shown on the right. On the left: P1, patient no. 1; P2, patient no. 2. Corresponding viral proteins (p) and glycoproteins (gp) are shown in the middle.

Table 1. Titers of Antibodies to EBV-EA and -VCA

	EVB-EA	EBV-VCA
Patient no. 1		
12/22/82	<1:5	1:10
5/30/83	<1:5	1:10
3/15/84	<1:5	1:40
9/12/84	<1:5	1:320
3/14/85*	1:10	1:1,280
3/10/86	<1:5	1:80
Patient no. 2		
12/07/85*	1:20	1:1,280
3/20/86	1:40	1:640
11/12/86		1:320

*At diagnosis of Burkitt's leukemia.

Bone marrow lymphoblasts from patient no. 2 were OKT11$-$, $\gamma+$, $\mu-$, $\delta-$, $\kappa-$ and $\lambda+$.

DNA hybridization studies. DNA extracted from the tumor cells was assayed by Southern blot analysis using as a probe a cloned HIV DNA fragment that contains the pol gene. No HIV-related sequences could be demonstrated (data not shown). Similarly, Southern blot hybridization to CMV, VZV, and HSV probes did not detect hybridizing sequences to any of them (data not shown). DNA hybridization with a ^{32}P-labeled EBV probe revealed the presence of EBV-specific sequences in DNA extracted from the tumors of both siblings (Fig 2).

Figure 3 depicts the Southern blot hybridization to a ^{32}P-labeled *c-myc* probe. In both DNA samples extracted from the tumor tissues, rearranged *c-myc* bands were found. In the tumor of both siblings, the rearranged *c-myc* bands were found in the *Hind*III digest but not in the *Eco*RI digest. The change in the length of the *Hind*III-*Hind*III *c-myc* fragment without alteration of the *Eco*RI-*Eco*RI *c-myc* fragment localizes the breakpoint to the *Eco*RI-*Hind*III fragment downstream to exon 3 of *c-myc* (Fig 3, insert).

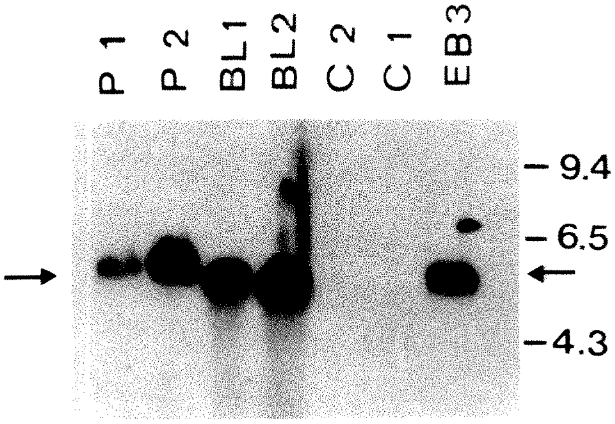

Fig 2. Detection of EBV sequences in DNA samples extracted from the patients' tumor cells. DNA samples were cleaved with *Bam*HI, subjected to electrophoresis through 0.7% agarose gel, and transferred to nitrocellulose paper. The paper was hybridized to a ^{32}P-labeled *Bam*K fragment of EBV. Hybridizing EBV sequences (arrows) were found in DNA samples from the patients' leukemic cells (P1 and P2) and in three EBV-positive Burkitt's lymphoma cell lines (BL1, BL2, and EB3). C1 and C2 are two negative controls. Numbers on the right are size markers in kilobases (bacteriophage λ, *Hind*III fragments).

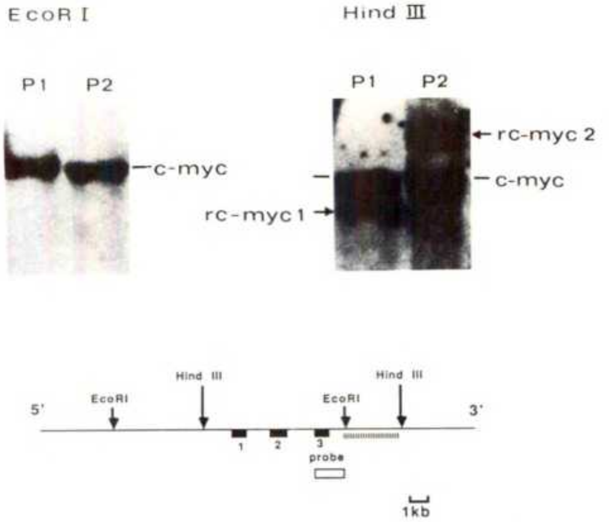

Fig 3. *C-myc* rearrangements in DNA samples from the patients' leukemic cells. DNA samples (15 μg) were digested by *Eco*RI (left) and *Hind*III (right). DNA fragments were separated by electrophoresis and transferred to nitrocellulose paper. The paper was hybridized to a ^{32}P-labeled *c-myc* probe. Hybridizing bands corresponding to *c-myc* germline genes are marked by dashes. Horizontal arrows indicate the rearranged *c-myc* genes detected in *Hind*III patient no. 1 (*rc-myc* 1) and patient no. 2 (*rc-myc* 2). The insert at the bottom depicts the restriction enzyme map of the human *c-myc* gene. The black rectangles 1, 2, and 3 correspond to exon 1, 2, and 3 of *c-myc*. Short vertical arrows point to the restriction sites of *Eco*RI and the long vertical arrows to the *Hind*III restriction sites. The interrupted line denotes the *Hind*III-*Eco*RI fragment that contains the breakpoints in both *rc-myc* genes. The open rectangle illustrates the *c-myc* probe (*ClaI-EcoRI* fragment containing exon 3).

Karyotype and HLA studies. Karyotype analysis was performed on the malignant cells of patient no. 2. A variant translocation t(2;8) was found (Fig 4), thereby suggesting the involvement of the chromosomal loci carrying the immunoglobulin κ light-chain genes and the cellular oncogene *c-myc*.[28,29]

The HLA phenotypes of the two affected brothers were identical: A11, A29, B53, B7, Cw2, Cw4, DR6, DR10.

DISCUSSION

The two hemophilic brothers developed AIDS-associated Burkitt's leukemia during the same year, and their tumor DNA contained EBV genomes and had rearranged *c-myc*. Both patients are still in complete remission 23 and 14 months, respectively. This favorable response in Burkitt's leukemia, especially in immunocompromised hosts, is surprising in view of the usually dismal course of the disease.[30]

In AIDS two factors may predispose the patients to B cell proliferation: HIV and EBV infections. It has been reported that HIV can cause direct B cell proliferation.[31] Furthermore, in some animal tumors, a direct role for the retroviral genome in the activation of cellular oncogenes has been demonstrated.[32,33] Therefore, we searched for HIV sequences in the patient's tumors and were able to confirm the absence of HIV genome in the tumors of both patients, as previously reported by Groopman et al and Pelicci et al.[34,35] It seems, therefore, that the HIV genome does not contribute directly to the malignant transformation.

On the other hand, the finding of the EBV sequences in the tumors suggests a role for this virus in the pathogenesis of AIDS-associated B cell lymphomas similar to other immunocompromised hosts.[36-40]

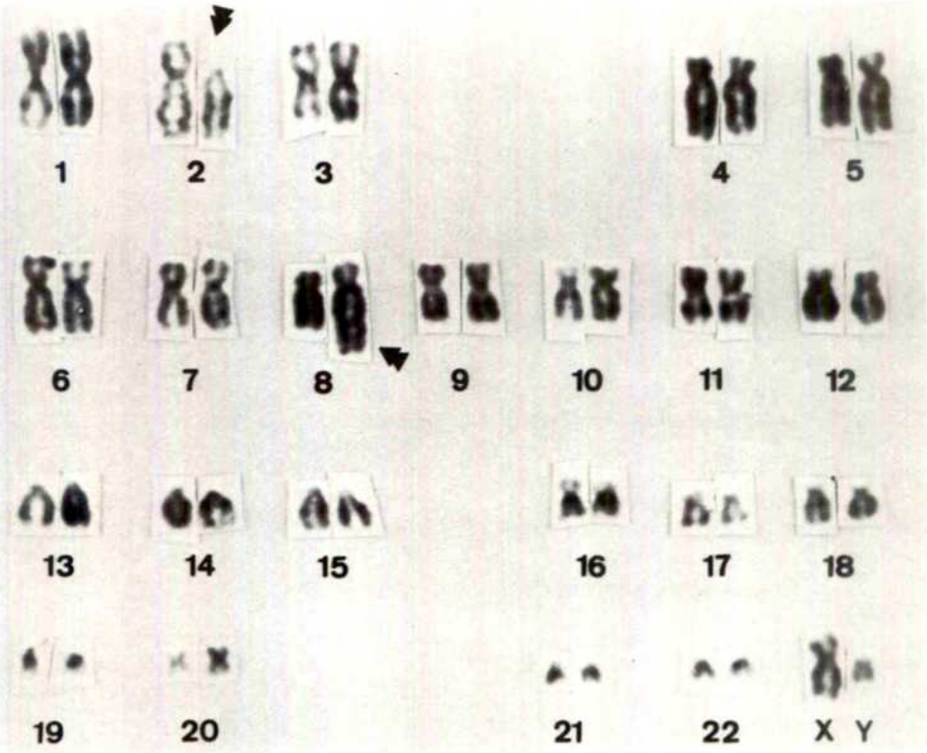

Fig 4. A representative karyotype of a bone marrow tumor cell from patient no. 2. The translocated chromosome 2 and 8 are indicated by arrows.

Most Burkitt's lymphomas analyzed thus far were found to carry a rearranged *c-myc* gene,[41,42] and the two tumors described here are no exception. In lymphomas carrying the classic t(8:14) translocation, the chromosomal breakpoints on chromosome 8 are 5′ to the two coding exons (2 and 3) of the *c-myc* oncogenes. In lymphomas carrying the variant translocations t(8:22) and t(2:8), the breakpoints are 3′ to exon 3 of *c-myc*. Our studies locate the breakpoints at 3′ of exon 3 of *c-myc*, thereby suggesting that in both our patients variant translocations are responsible for the *c-myc* activation. Karyotype analysis of the malignant cells of patient 2 indeed revealed the existence of t(2:8). Such variant translocations are found only in a minority of Burkitt's lymphomas, and their occurrence in the reported two brothers is interesting. Cytogenetic studies have been performed only in a small number of AIDS-associated lymphomas, and three cases of the variant t(8:22) translocation were reported in such tumors.[43-46] Analysis of more lymphomas from AIDS patients are necessary to conclude whether the occurrence of variant translocations is more prevalent in this group. The pattern of antibodies to EBV in our patients as well as other immunocompromised hosts is consistent with reactivation of a latent virus. In African Burkitt's patients, tumors develop at a young age where the expanded pool of activated B cells at risk for *c-myc* rearrangement is composed mainly of IgM-producing lymphocytes. Therefore, the majority of Burkitt's lymphomas express IgM.[47] In the patients with AIDS, as expected in secondary response, there is a large pool of IgG-transformed lymphocytes, and therefore it is not unexpected that some of the tumors will be IgG producers as in our cases.

It has been shown by Lenoir et al[48] that Burkitt's tumors and cell lines carrying the t(2:8) translocation usually express κ light chains whereas those with the t(8:22) translocation synthesize λ light chains. It was suggested, therefore, that the type of *c-myc* transposition is in correlation with the level of differentiation of the B cell.[48] Lymphoblasts of patient no. 2 are unusual in that they carry t(2:8) but express λ light chains. Similarly, Magrath et al reported that the lymphoma cells of a homosexual with AIDS and t(8:22) expressed κ light chains.[43] Taken together, the expression of IgG, the possibly higher incidence of variant translocations, and the lack of correlation between the type of light-chain expression and type of translocation suggest that the sequence of events resulting in B cell tumors in these patients may be different from that of African Burkitt's lymphomas. The two brothers reported on here had an identical HLA phenotype, and although this could have been a mere coincidence, it is tempting to speculate that genetic factors played an important role in the infection with HIV[49-51] and in the evolution of the lymphatic malignancy.[52-54]

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Incidence of Aplastic Anemia: The Relevance of Diagnostic Criteria

By the International Agranulocytosis and Aplastic Anemia Study

The incidence of aplastic anemia was estimated in a 4-year study conducted in Israel and seven areas in Europe. Strict definition criteria were used, and all data and bone marrow specimens were reviewed by a panel of experienced hematologists. Complete ascertainment of cases was attempted by establishing a telephone network including all relevant hospitals in the study areas. The incidence of aplastic anemia was estimated to be less than three per million per year, a figure lower than previously reported. The most likely explanation for the differences among studies is variation in diagnostic criteria, which might lead to an overestimation of the incidence in some previous studies.

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APLASTIC ANEMIA is a rare but serious blood dyscrasia that may be induced by environmental factors such as drugs or viral infections. There are only limited data on its incidence, which appears to vary according to the period of observation and geographic area.[1-6] It is possible that the variation is due to genetic differences and to varying prevalences of exposure to potentially myelotoxic agents. However, methodological problems such as the completeness of case ascertainment or stringency of diagnostic criteria may also be responsible for at least some of the differences observed. In this paper we report incidence data obtained in a multicenter study of agranulocytosis and aplastic anemia that was carried out from 1980 to 1984 in Israel and seven regions of Europe. The aim of the study was to quantify associations among drugs, especially antiinflammatory and analgesic agents, and two blood dyscrasias, agranulocytosis and aplastic anemia.

METHODS

The methods of the study have been described.[7] Here the description is limited to aspects of particular relevance to estimating the incidence of aplastic anemia. The study base included the entire population of eight defined geographic regions: Ulm, FRG; Berlin; Barcelona, Spain; Milan, Italy; Budapest; Sofia, Bulgaria; Stockholm/Uppsala; and Israel. The total population was 20.5 million, as determined from census data obtained from official national sources. Case ascertainment began in July 1980 and ended in June 1984 (not all regions participated for the full 4-year period).

Efforts were made to identify all cases of aplastic anemia occurring in the regions. A study center in each region obtained information on potential cases from hospitals within a defined area; these hospitals included all clinical institutions known to treat diseases of the severity of aplastic anemia. The study centers maintained regular liaison with the cooperating hospitals at intervals of no more than 2 weeks. In each hospital, an appropriate individual, usually a hematologist or technician, was contacted by telephone to ascertain all cases of aplastic anemia. Once notification of a potential case was received, a specially trained nurse or physician monitor was sent to collect more detailed clinical information and histopathologic specimens. A consulting hematologist reviewed the information in each of the eight regional centers. If the case clearly did not comply with the definition of aplastic anemia as required by the protocol, it was excluded locally. All remaining potential cases were reviewed subsequently by the Hematology Review Committee (HRC, see the Appendix) using clinical summaries, hematologic data, and microscopic examination of bone marrow and peripheral blood specimens. This panel had no information on previous exposures to drugs or chemicals.

For confirmation of diagnosis, the following conditions were obligatory: peripheral blood (at least two of the following three criteria): (*a*) hemoglobin, ≤ 100 g/L, or hematocrit, $\leq 30\%$; (*b*) platelets, $\leq 50 \times 10^9$/L; and (*c*) leukocytes $\leq 3.5 \times 10^9$/L, or granulocytes, $\leq 1.5 \times 10^9$/L. For bone marrow to confirm the diagnosis, there had to be an adequate bone marrow biopsy specimen or histology specimen obtained at autopsy showing the following: (*a*) a decrease in cellularity with the absence or depletion of all hematopoietic cells or normal cellularity due to focal erythroid hyperplasia with depletion of granulopoietic cells and megakaryocytes and (*b*) the absence of significant fibrosis or neoplastic infiltration. Cases with pancytopenia due to cytostatic drugs or irradiation were excluded.

If there were equivocal data that did not permit a definite diagnosis, the following additional information was sought from the local hematologist for second review: follow-up blood counts including reticulocyte counts, subsequent bone marrow specimens, and autopsy reports. If one of the two peripheral blood criteria to be fulfilled was hemoglobin, ≤ 100 g/L, or hematocrit, $\leq 30\%$, a reticulocyte count of less than 30×10^9/L was required.

All patients were observed until death, recovery, or bone marrow transplantation, or for a period of at least 2 years.

RESULTS

There were 315 cases initially identified as aplastic anemia and four cases originally identified as agranulocytosis but subsequently confirmed as aplastic anemia. Of the 319 potential cases, 34 were clearly misdiagnosed and were therefore ruled out by the consulting hematologists at the local centers (Fig 1). Among the 285 potential cases reviewed by the HRC, 168 were confirmed as definite aplastic anemia; nine cases were included even though a bone marrow biopsy specimen was not available because at least two bone marrow aspirations were typical and all clinical data over a prolonged period of observation were compatible with the diagnosis. Among the potential cases excluded by the HRC were 40 patients in whom a definite diagnosis could not be made due to insufficient documentation, in the majority due to the absence or inadequate technical quality of the biopsy specimen. Seven cases of moderate pancytope-

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From the International Agranulocytosis and Aplastic Anemia Study. See the Appendix for a complete list of participants.

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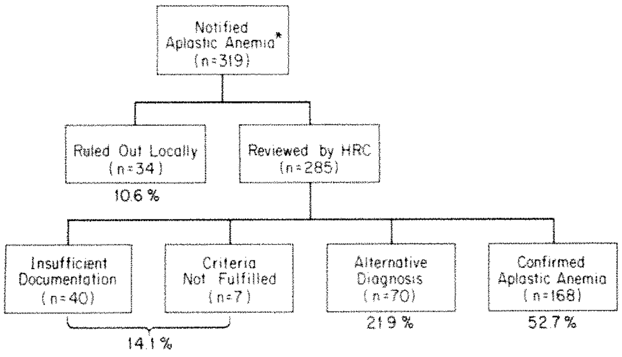

Fig 1. Initial diagnosis of aplastic anemia and results of expert review. *Includes four cases that were diagnosed as agranulocytosis but confirmed as aplastic anemia.

nia did not fulfill the minimum criteria; in these patients, the bone marrow showed only patchy or borderline hypocellularity.

There were 70 potential cases identified as having pancytopenia due to another disease. The largest group (17 cases) consisted of pancytopenia with normal or hypercellular bone marrow that could not be further classified from the data available to the HRC; ten of these were recognized as transient pancytopenia on further follow-up. In 13 patients, a definite diagnosis of leukemia could not be made, but increased cellularity of the bone marrow and morphological aberrations of myelopoietic precursors permitted their identification as myelodysplasias that were potentially preleukemic. Nine cases were identified as aleukemic myeloid or myelomonocytic leukemia, usually of an oligoblastic or smouldering type. There were seven cases with severe neutropenia and additional anemia or thrombocytopenia; review of the bone marrow biopsy specimens, details of clinical presentation, and subsequent patterns of blood counts revealed that the anemia or thrombocytopenia was due to secondary events such as bleeding or infection and that the correct diagnosis was agranulocytosis or chronic neutropenia. Non-Hodgkins malignant lymphoma involving the bone marrow (including two cases of aleukemic hairy cell leukemia) was identified in six cases. Five cases were determined to have hypersplenism or liver disease, and the remaining 13 cases included diagnoses such as megaloblastic anemia, chronic renal disease, and multiple myeloma, none of which accounted for more than three individuals.

The overall incidence of aplastic anemia, based on the total of 166 complete and confirmed cases, was estimated to be 2.2 cases per million per year (Table 1). (Data from Stockholm/Uppsala are not included in the overall incidence rates because clot sections from aspirated bone marrow spicules are often taken instead of a core biopsy, thereby resulting in a larger proportion of potential cases in that region being excluded due to insufficient documentation in terms of the study criteria.) The region-specific incidence estimates ranged from 0.6 per million in Budapest (based on only three cases) to 3.1 per million in Milan. A test for heterogeneity in the region-specific rates among the five centers contributing at least 18 cases was not statistically significant.

Table 1. Annual Incidence of Aplastic Anemia According to Region, July 1980 to June 1984

Region	Population (10^6)	Interval (mo)	n	Incidence (per 10^6/yr)*
Ulm	5.3††	48	56	2.8
Berlin	1.8	48	18	2.2
Barcelona	4.1	48	36	2.2
Israel	3.9	48	21	1.6
Milan	2.3	45†	27	3.1
Budapest	2.0	31‡	3	0.6
Sofia	1.1	23§	5	2.6
Stockholm/Uppsala	1.8	18‖	2	—¶
Total	20.5#	—	—	2.2**

*Standardized to the age and sex distribution of the study population.

†October 1980 to June 1984.

‡December 1981 to June 1984.

§August 1982 to June 1984.

‖January 1983 to June 1984.

¶The incidence was not calculated for Stockholm/Uppsala for methodological reasons (see the text).

#The total population does not include Sweden.

**Calculated as a population- and time-weighted average of the age- and sex-standardized region-specific rates.

††Including a large region surrounding the city.

The incidence according to age and sex is given in Table 2. The sex ratio for all regions combined was close to unity. Among females, the incidence of aplastic anemia increased steadily with age to a peak of 6.3 per million above the age of 60. Among males, the highest incidence was in the 15- to 24-year-old age group. However, nine of the 22 cases were from one military brigade in Barcelona; this result will be reported more fully elsewhere. If the latter age group is ignored, the incidence in males was highest (3.2 per million) over the age of 60 years. No attempt was made to compare age or sex-specific incidence rates between the study regions due to the limited number of cases. The fatality rate 2 years after diagnosis was 49%.

DISCUSSION

The overall incidence of aplastic anemia as estimated in this study is considerably lower than both incidence and mortality estimates obtained from other studies.[1-6,8-11] Aoki et al[1] and Hayakawa and Kurihara[3] used death certificate statistics to compare mortality rates in different regions.

Table 2. Annual Incidence of Aplastic Anemia According to Age and Sex

	Male		Female	
Age (yr)	n	Incidence (per 10^6/yr)*	n	Incidence (per 10^6/yr)*
<15	12	1.7	5	0.7
15-24	22†	3.5	4	0.9
25-44	12	1.1	18	1.8
45-59	11	1.8	15	2.0
≥60	16	3.2	51	6.3
Total	73	2.1	93	2.4

*Standardized to the regional distribution of the study population.

†Includes nine cases from Barcelona.

High rates for aplastic anemia were reported from Japan, compared with most European countries and the United States. It is unknown what proportion of deaths attributed to aplastic anemia in these reports were actually aplastic anemia. In the studies of Wallerstein et al[6] and Szklo et al,[5] outpatient and hospital charts, autopsy records, or laboratory material was used to confirm the diagnosis in cases selected by a review of death certificates. In no more than 60 of 290 and 60 of 111 cases, respectively, could aplastic anemia be confirmed, although it is possible that a proportion of those who failed to satisfy study criteria lacked sufficient documentation. Even larger discrepancies may exist in countries with less adequate diagnostic facilities than in the United States. Uncontrolled mortality statistics, therefore, give at best only a very rough estimate of the true incidence of the disease.

Even the highest regional incidence in the present study (3.1 per million in Milan) is lower than previous estimates, which range from 4 to 25 cases per million per year (Table 3). The latter figures are based on data derived from both mortality and morbidity statistics collected in different regions and at different times. Potential methodological problems and other possible explanations must be considered in interpreting the apparent differences.

The estimate of Wallerstein et al[6] was obtained in the course of quantifying the risk of aplastic anemia in relation to exposure to chloramphenicol; incidence estimates were derived from mortality data, assuming a 50% disease-specific death rate. The limitations of death certificate data have already been mentioned. In addition, it is not clear that the assumption of a 50% death rate is correct. We observed a similar death rate in our series after only 2 years of follow-up, and it is probable that the rate would have been higher with a longer follow-up. Thus, the total incidence could have been overestimated by Wallerstein et al.

It is important to consider whether the lower incidence of aplastic anemia observed in our study, and based on recent data, reflects a true decrease over the last 20 years (for example, because of decreased use of potentially harmful drugs such as chloramphenicol). This explanation seems unlikely because no clear trend has been observed in mortality statistics from different areas between 1950 and 1980[3] or between 1970 and 1979 in a study by Szklo et al using morbidity data.[5]

It also does not seem likely that differences in age between the study populations can completely account for the observed differences in incidence. Presumably, the age distributions in the study regions have not changed materially over time; if anything, the populations have aged, thus increasing rather than decreasing the incidence of a disease with the age dependency of aplastic anemia. Evidence against differences in age distributions was suggested by a comparison of the study population of Szklo et al[5] with our data: the age and sex characteristics of the population of metropolitan Baltimore were virtually identical to those in our study.

There is no evidence that the lower incidence in our study is due to lower ascertainment than in previous studies. Utmost care was taken to obtain regular information from all the cooperating hospitals in the telephone network. Although it is possible that some patients with aplastic anemia from the defined geographic areas had been referred to hospitals outside of these areas, this is also true for the other studies listed in Table 3. There were 47 cases excluded because of insufficient documentation or not fulfilling our study criteria; if the assumption is made that all of those excluded were actually cases of aplastic anemia, the incidence rate would only have increased from 2.2 to 2.8 per million per year.

We suggest that the major reason for the differences observed between the present study and those listed in Table 3 is differences in the stringency of diagnostic criteria among studies. Quantitative estimation of bone marrow cellularity is of paramount importance for the diagnosis of aplastic anemia, as against other blood dyscrasias resulting in pancytopenia. This is particularly relevant for the differential diagnosis between aplastic anemia and the various myelodysplastic syndromes. An adequate bone marrow biopsy specimen is therefore regarded as mandatory for a definitive diagnosis of aplastic anemia[8,9,12] and is a requirement for inclusion in therapeutic trials.[13,14] In this study, the results of the review by the HRC of the cases originally diagnosed as aplastic anemia, including an examination of blood films and bone marrow specimens, demonstrated that it is not sufficient to rely on the diagnosis as derived from hospital reports. The primary reason for the exclusion of potential cases was a bone marrow specimen of insufficient quantity or quality or an inadequate histopathologic evaluation. Only 5% of the cases were accepted without a bone marrow biopsy. Biopsy was not required in the studies of Wallerstein et al,[6] Böttiger and Westerholm,[10] or Szklo et al[5]; and in the study of Modan et al[4] there was no bone marrow examination at all. Thus, it appears that pancytopenias other than aplastic anemia may have been included as true aplastic anemia in previous studies and that the incidence estimates were erroneously high.

Table 3. Incidence Estimates of Aplastic Anemia From Various Studies

Author	Study Period	Area	Population $(\times 10^6)$	n	Incidence (per 10^6/yr)
Modan et al[4]	1961-1965	Israel	2.4	93	7.8
Wallerstein et al[6]	1963-1964	California	17.5	60	4.8
Böttiger et al[10]	1964-1968	Sweden	1.2	80	13
Szklo et al[5]	1970-1978	Baltimore	1.6*	62	4.1*
Böttiger et al[11]	1973-1977	Sweden	1.3	157	25
Present series	1980-1984	Europe/Israel (see Table 2)	20.5	166	2.2

*White population only.

Our criteria confined the definition of aplastic anemia to severe and moderate cases; borderline cases, of somewhat less concern due to good prognosis and low mortality, were not included. Such cases may have been included in other series, but this also cannot explain completely the differences shown in Table 3. Blood counts were described as "pancytopenia" or "severe panctyopenia" in the reports of Böttiger et al[10,11] and Wallerstein et al,[6] respectively. Upper thresholds for hematocrit and neutrophil values as indicated by Szklo et al[5] and Modan et al[4] were similar to ours.

The most striking difference exists between our data and the incidence estimates of Böttiger et al.[10,11] According to the detailed information in their more recent paper,[11] they have included cases of bone marrow failure other than aplastic anemia such as hematopoietic neoplasia, myelofibrosis, or pancytopenia due to cytostatics. Exclusion of these cases would reduce the incidence rate from 25 to 12.3 cases per million per year. Because only 10% of the cases of "true" aplastic anemia are attributed to drugs other than cytostatics,[11] differences in diagnostic criteria rather than different drug exposure rates may explain the discrepancy between Böttigers' data and those reported here and from the Baltimore area.[5]

The possibility of misclassification of a substantial fraction of cases may have important implications for the validity of data concerning the drug etiology of aplastic anemia (as well as the effects of other chemicals) that are derived from many previous reports. Most hematologists would agree that in practice, knowledge of exposure to potentially myelotoxic drugs can often influence the physician in making a diagnosis of aplastic anemia when a patient has pancytopenia. This could result in bias due to selective reporting of exposure-positive pancytopenia as drug-induced aplastic anemia irrespective of whether the diagnosis is correct.

In conclusion, we suggest that the lower incidence reported in our study is closer to the actual incidence than the estimates obtained from previous studies. Revisions of the commonly accepted assumptions of risk due to environmental factors may therefore be necessary.

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APPENDIX

Participants are as follows: Chairman, M. Levy, Jerusalem; Coordination and Data Analysis (Slone Epidemiology Unit), D. Slone,† S. Shapiro, D.W. Kaufman, T. Anderson, L.J. Blair, P.T. Cook, J.J. Farrell, L.F. Gaetano, R. Matis, J.P. Kelly, C.L. Wells, Boston; Investigators, J.R. Laporte, Barcelona, Spain; H. Kewitz, Berlin; G. Retsagi, S. Hollan, Budapest; M. Levy, Z. Stern, Jerusalem; G. Tognoni, Milan, Italy; V. Vlahov, Sofia, Bulgaria; B.E. Wiholm, Stockholm; H. Heimpel, W. Heit, Ulm, FRG; Data Collection, X. Carne, L. Ibanez, Barcelona, Spain; M. Klein, C. Scott, Berlin; E. Czink, A. Laszlo, Budapest; M. Beiski, F. Simon, Z. Toledano, Jerusalem; C. LaVecchia, P. Liati, M.G. Zurlo, Milan, Italy; N. Bacracheva, Sofia, Bulgaria; K. Jacobsen, M. Sandberg, Stockholm; I. Bayer, R. Kreuzer, Ulm, FRG; Hematology Review Committee, H. Heimpel, W. Heit, Ulm, FRG; G. Lambertenghi-Deliliers, Milan, Italy; A. Polliack, Jerusalem. The study is supervised by an Honorary International Advisory Board whose members are Sir Richard Doll, FRCP, FRS, Oxford, Chairman; Per Knut M. Lunde, MD, Oslo; and Sven Moeschlin, MD Solothurn, Switzerland.

†Deceased.

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Monoclonal Antibody–Specific Immobilization of Platelet Antigens (MAIPA): A New Tool for the Identification of Platelet-Reactive Antibodies

By V. Kiefel, S. Santoso, M. Weisheit, and C. Müeller-Eckhardt

The analysis of sera containing different platelet-reactive antibodies, eg, autoantibodies, platelet-specific alloantibodies like anti-Pl^{A1}, -Pl^{A2}, -Bak^{a}, and HLA antibodies, is still difficult. Recently, monoclonal antibodies against major platelet membrane constituents (glycoproteins IIb/IIIa and Ib and HLA class I molecule) have become available. In this report we describe a new assay that takes advantage of these highly specific reagents to investigate selectively platelet reactive antibodies against epitopes on different glycoproteins. The reliability and specificity of this assay is demonstrated with known platelet-reactive autoantibodies and alloantibodies (anti-Pl^{A1}, -Bak^{a}, -Pen). The discovery of a Pl^{A2} antibody in a serum of a polytransfused patient underscores the efficiency of this technique. Possible applications of this assay are discussed in detail.

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IMMUNE-MEDIATED PLATELET DESTRUCTION in conditions such as neonatal alloimmune thrombocytopenia, posttransfusion purpura, platelet transfusion refractoriness, and autoimmune thrombocytopenia is caused by a variety of platelet-reactive antibodies. Their serological dissection is essential for proper diagnosis and treatment. Of particular importance is the discrimination between platelet-specific antibodies, ie, anti-Pl^{A1} (anti-Zw^{a}), anti-Bak,^{a} and HLA antibodies that are frequently encountered in the same serum specimens. Of similar interest is the assignment of antigenic determinants to certain platelet membrane constituents. Thus, known platelet-specific antigens reside on the glycoprotein complex IIb/IIIa (GP IIb/IIIa) ie, Pl^{A1},[1] PEN,[2,3] Yuk^{a} and Yuk^{b}[4,5] on GP IIIa and Bak^{a} (Lek^{a}) on GP IIb,[6,7] whereas in several cases of chronic immune thrombocytopenia autoantigens have been localized on the GP IIb/IIIa complex or on GP Ib.[8-12]

Presently available techniques for antibody differentiation and/or antigen recognition use stripping of HLA antigens from platelets by chloroquine pretreatment,[13,14] solid-phase radioimmune assay,[8,9] or Western blotting procedures.[15] All these assays have their limitations in that they either are indirect (chloroquine method), destroy HLA antigens (Western blotting), or are insensitive due to high background values (solid-phase radioimmune assay).

Therefore we have developed an enzyme immunoassay that overcomes most of these difficulties. It takes advantage of the selective specificity of platelet-specific monoclonal antibodies and achieves high sensitivity by avoiding direct contact between the serum to be investigated and the solid phase. In this report we give a detailed description of the method and demonstrate some of its applications.

MATERIALS AND METHODS

Human antibodies. Six human sera containing various platelet-reactive antibodies were selected for this study. Their origin and predetermined specificities are listed in Table 1. All sera had been prescreened against lymphocytes and platelets from a large panel of donors with known HLA, PL$^{A1/A2}$, and Bak^{a} antigens by the lymphocytotoxicity test (LCT),[16] the platelet complement fixation test (PCFT),[17] and the platelet adhesion immunofluorescence test (PAIFT).[18]

Monoclonal antibodies. The following murine monoclonal antibodies (MoAb) were used: MoAb Gi5, raised and characterized in our laboratory,[19] directed against the human platelet GP IIb/IIIa complex; MoAb FMC25, a gift from Dr Zola, Adelaide, Australia, specific for GP IX,[20] a glycoprotein tightly complexed to GP Ib; and MoAb w6.32 specific for a monomorphic epitope on the heavy chain of HLA class I molecules.[21]

MoAb-specific immobilization of platelet antigens (MAIPA). Platelets were isolated by differential centrifugation from EDTA-anticoagulated blood and washed three times. A volume of washed platelets, stored at 4°C for at least 12 hours in isotonic saline containing 0.1% sodium azide, was pelleted to give a total of 1×10^{8} platelets. The pellet was resuspended in 50 μL phosphate-buffered saline (PBS), pH 7.2, supplemented with 2% bovine serum albumin (BSA). Ten to 40 μL of a platelet glycoprotein–specific MoAb diluted at a concentration of 0.02 mg/mL in PBS-BSA, and 5 to 200 μL of the serum to be investigated was added. The mixture was incubated at 37°C for 30 minutes. The platelets were then washed three times in isotonic saline and solubilized in 100 μL of 0.01 mol/L Tris-buffered saline (TBS) containing 0.5% Nonidet P40 for 30 minutes at 4°C. Then all samples were centrifuged at 15,000 g for 30 minutes at 4°C. Fifty microliters of the supernatants was diluted 1:5 in TBS wash buffer (TBS with 0.5% Nonidet P40, 0.05% Tween 20, and 0.5 mmol/L $CaCl_2$). One hundred microliters of the respective diluted supernatants was added to each well of a microtiter tray (Greiner, Nürtingen, FRG) that had been coated with 100 μL goat antimouse IgG (Dianova, Hamburg, FRG; final antibody concentration, 3 μg/mL in 0.05 mol/L carbonate buffer) by overnight incubation at 4°C, washed, and blocked for 15 minutes at 4°C with 200 μL TBS wash buffer per well. The trays were incubated for 90 minutes at 4°C and washed four times, and 100 μL alkaline phosphatase–labeled goat antihuman IgG (Fc) (Dianova) diluted 1:5,000 in TBS wash buffer was added. After incubation for 120 minutes at 4°C the tray was washed six times, and 100 μL substrate solution (paranitrophenylphosphate in diethanolamine buffer, pH 9.8) was added. The color reaction was stopped after 30 minutes with 3 N NaOH and read at 405 nm in a Titertek photometer. All tests were run in duplicate. Results were expressed as ΔE values, ie, the difference of extinction (optical density) values between the mean of test samples and blanks (wells devoid of platelet lysate). A positive color reaction indicates a reaction of a human antibody with an epitope on the same molecule recognized by the MoAb. Reactions with other membrane constituents give no visible signal.

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Table 1. Clinical and Serological Data of Patients With Platelet-Reactive Antibodies

Serum No.	Clinical Condition of Serum Donor	PAIFT Antibody Specificity	PAIFT Titer	LCT	PCFT	Comments
1	Posttransfusion purpura	Anti-Pl^{A1}	4/5* 1:64†	1/29*	neg	Pl^{A1}-negative
2	Healthy mother of child with NIT	Anti-HLA (polyspecific)	5/7*	22/25*	7/19*	Mother, Pl^{A1}-positive
3	Autoimmune thrombocytopenia	Autoantibodies	5/5* 1:128†	neg	neg	Antigenic determinant on GP Ib (immunoblotting)
4	Healthy mother of child with NIT	Anti-Pen	7/7* 1:32†	neg	nt	Kindly provided by Dr R.H. Aster (Milwaukee)
5	Posttransfusion purpura	Anti-Bak^a + anti-HLA	1:32‡ 1:128§	22/30*	neg	Kindly provided by Dr A. Waters (London)
6	Malignant mastocytosis; multiple blood transfusions	Anti-HLA (polyspecific)	5/5* 1:128†	29/29*	Complement inhibition	Pl^{A1} antigen–positive; RBC antibodies anti-Jk^a, -C^w, -S

Sera were characterized with PAIFT, LCT, and PCFT.

Abbreviations: nt, not tested; neg, negative; RBC, red blood cells.

*Number of positive cells per number of cells tested.

†Titer.

‡Titer when tested with Bak^a-negative cells.

§Titer when tested with Bak^a-positive cells.

Inhibition of calcium-dependent proteases in platelet lysates has no effect on antibody assessment. In experiments using leupeptin and sera no. 1 and 3, the results with platelets from four donors were identical whether platelets were lysed in the presence (1 mg/mL) or absence of leupeptin.

The intraassay and interassay reproducibility of results is very satisfactory. Representative figures ($\bar{x}$ ± 1 SD) for an antibody-containing serum compared with a negative control serum with platelets from the same donor were as follows: intraassay variability, 0.913 ± 0.041 *v* 0.05 ± 0.017 (n = 8); and interassay variability, 1.426 ± 0.240 *v* 0.06 ± 0.015 (n = 8).

RESULTS

Glycoprotein assignment of platelet antibodies. In a first series of investigations, the antigenic determinants on different platelet membrane molecules of operationally "monospecific" platelet antibodies were evaluated. The results of representative experiments are illustrated in Figs 1 and 2. When serum 1 containing pure Pl^{A1} antibodies was assessed against a panel of platelets with different Pl$^{A1/A2}$ types, it reacted only with determinants on GP IIb/IIIa complexes of Pl^{A1}-positive but not PL^{A1}-negative platelets if MoAb Gi5 was used for immobilization. No reactivity was noted with molecules carrying HLA determinants immobilized with MoAb w6.32 (Fig 1). In contrast, serum 2, which solely held multispecific HLA antibodies, was strongly positive with HLA determinants immobilized by MoAb w6.32, irrespective of the PL^{A} type of panel platelets used. There was no reactivity with GP IIb/IIIa determinants.

In Fig 2, the discriminatory capacity of the MAIPA with regard to epitopes on different platelet glycoproteins is demonstrated. Three platelet-specific antibodies (sera 1, 3, and 4) were assayed against antigens either on the GP IIb/IIIa complex or on the GP Ib complex, which were immobilized by MoAbs Gi5 or FMC25, respectively. Although serum 1 (known Pl^{A1} antibodies) reacted only with determinants of Pl^{A1}-positive donors on the GP IIb/IIIa complex but not on the GP Ib complex, the reverse pattern was seen with serum 3. Immunoblotting studies had shown

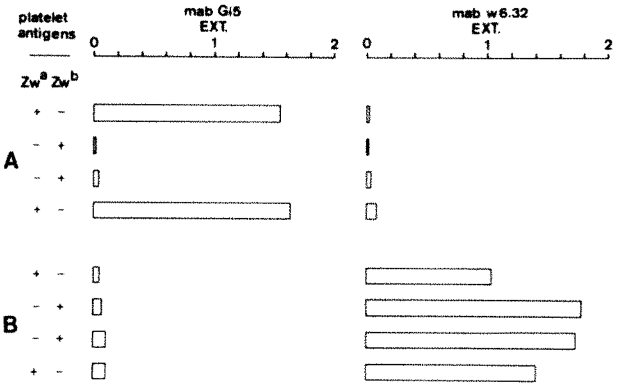

Fig 1. Reaction of a platelet-specific alloantibody, anti-Pl^{A1} (Zw^a), with an antigen present on Pl^{A1}-positive cells immobilized by MoAb Gi5 (A) and of a polyspecific HLA antibody with an antigen on the platelet surface immobilized by MoAb w6.32 (B). Ext, extinction: optical density at 405 nm.

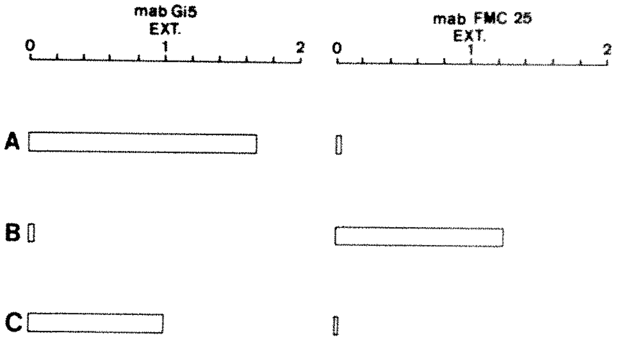

Fig 2. Specific reactions of platelet alloantibodies with the GP IIb/IIIa complex: anti-Pl^{A1} (A), anti-Pen (C) and of a platelet autoantibody (B) reacting with the GP Ib complex immobilized by MoAb FMC25.

that this serum contained only platelet autoantibodies reacting with GP Ibα specificity.[12] This was confirmed by MAIPA. Serum 4 (kindly provided by Dr R.H. Aster, Milwaukee) had Pen antibodies that reacted in PAIFT with a high-frequency platelet antigen. It detected antigenic determinants present on the GP IIb/IIIa complex (immobilized by MoAb Gi5) but not on the GP Ib complex (immobilized by FMC25), which confirmed the results of Furihata et al.[3]

Dissection of mixtures of platelet-reactive antibodies. Two typical examples are depicted in Fig 3. Serum 5, a gift from Professor A. Waters (London), was known to contain high-titered multispecific HLA antibodies (positive with 29/29 panel cells in LCT) and, in addition, Bak^a antibodies. In PAIFT, no definite distinction of these two types of antibodies was possible at lower dilutions. When using the MAIPA, both antibody specificities were clearly discernible: the Bak^a antibodies reacted in an antigen-specific fashion with their epitope on GP IIb/IIIa immobilized by MoAb Gi5, whereas the HLA antibodies were positive with MoAb w6.32–immobilized HLA antigens.

Serum 6 was unusual in that it so far had been considered to contain only strong multispecific HLA antibodies. On MAIPA analysis, an additional Pl^{A2} antibody was detected that reacted with MoAb Gi5–immobilized GP IIb/IIIa determinants in a Pl^{A2}-specific pattern. The Pl^{A2} specificity was further confirmed in dosage determinations by using platelets of Pl^{A2} homozygous and $Pl^{A1/A2}$ heterozygous donors (data not shown).

DISCUSSION

The identification of platelet-reactive antibodies is still a notoriously difficult task. This is particularly due to the fact that most sera contain mixtures of antibodies. Because the majority of patients with immune platelet disorders are women and have been pregnant and/or received blood or platelet transfusions, they are often immunized against HLA-A, -B antigens. For instance, eight of 14 patients with posttransfusion purpura we thus far have diagnosed had strong lymphocytotoxic antibodies in conjunction with Pl^{A1} antibodies, and in the serum of one patient, HLA antibodies were identified exclusively.[22] Recent reports on patients with posttransfusion purpura due to rare antibody specificities, ie, anti-Bak^a,[23] anti-Lek^a,[24] and anti-Pl^{A2},[25] have indicated that these sera were also heavily contaminated with HLA antibodies. In patients receiving multiple platelet transfusions it is estimated that the refractory state of approximately 25% to 30% of patients is caused by platelet-specific antibodies, although their specificity has not been identified.[26]

Some progress in differentiating HLA from other platelet-reactive antibodies has recently been made by the method of chloroquine stripping of HLA antigens from platelets.[13] By this technique, Nordhagen and Flaathen[14] successfully discriminated HLA from Pl^{A1} and Bak^a antibodies by using the platelet suspension immunofluorescence test. Unfortunately, chloroquine treatment can be applied only to fresh platelets and is fraught with the disadvantage of unspecific uptake of the fluorescent dye after cell damage. In our hands, weak platelet-specific antibodies often escape identification by this technique.

Another approach to antibody identification is the use of immunoblotting techniques.[15,27] Although some platelet-specific alloantigens and autoantigens can readily be demonstrated,[6,7,10-12,28] HLA-A, -B antigens and possibly other as yet unidentified antigens are often destroyed by sodium dodecyl sulfate treatment of the platelet lysate. This is corroborated by the observation that some monoclonal platelet antibodies fail to bind to immunoblots. Moreover, the use of panels of several solubilized platelet suspensions in immunoblotting is laborious and time-consuming, and its results are difficult to interpret.

The microtiter assay described by Woods et al[8,9] used MoAbs fixed to a solid phase. It allowed us to demonstrate platelet autoantigens on GP IIb/IIIa or GP Ib, respectively. However, in our experience this assay is rather insensitive, most likely as a consequence of low binding ratios. Even strong Pl^{A1} antibodies yielded rather low binding ratios that were apparently caused by high background activity due to unspecific adsorption to the solid phase of components to be investigated.

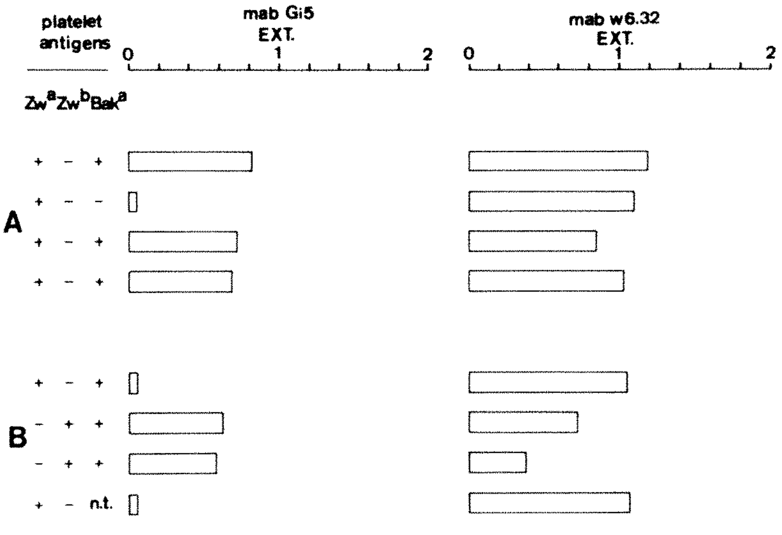

Fig 3. **Analysis of sera containing mixtures of platelet-specific alloantibodies and HLA alloantibodies with a panel of known donor platelets. When using MoAb Gi5 for immobilization, platelet alloantibodies anti-Bak^a (A) and anti-Pl^{A2} (anti-Zw^b) (B) were detected through their typical pattern of reactivity, whereas the antigens immobilized by MoAb w6.32 carried HLA alloantibodies present in the same samples.**

Antibody detection by MAIPA as described here circumvents most of these difficulties. It is based on the detection of trimolecular complexes formed by a MoAb, the human platelet-reactive antibody, and a platelet membrane molecule carrying the respective epitopes of either antibodies. Because antibody binding of both the monoclonal and the human antibody occurs on the surface of the intact platelet before solubilization, all antigens are preserved. Washing platelets after the sensitization phase removes excess MoAb as well as unbound serum. This ensures specific immobilization of the MoAb-platelet antigen complexes to the solid phase as well as specific binding of the labeled antibody to the human immunoglobulins associated with them. Hence, the background is usually low, and therefore, the specific binding values (difference between test and blank sample) are high. By using a set of MoAbs for different membrane constituents and a platelet panel with known alloantigens, platelet-reactive antibodies can be characterized in a single experiment with regard to both localization of the epitopes (eg, HLA class I molecule, GP IIb/IIIa complex, GP Ib complex, ie, GP Ib and GP IX) and the serological specificities. A possible false-negative result might be expected if monoclonal and human antibody have the same or a closely adjacent epitope for which they compete and therefore mutually hinder their binding. In these cases a MoAb directed against a different epitope on the same molecule is recommended. However, we have not observed such an interference between MoAb Gi5 and the platelet alloantigens tested thus far (Pl^{A1}, Pl^{A2}, Bak^{a} (Lek^{a}), Pen, Yuk^{a}, Yuk^{b}). Other advantages of this assay relate to its simplicity: unfixed platelets may be stored for weeks at 4°C without a loss of reactivity. Performance of the assay only requires six to seven hours. No sophisticated equipment is necessary.

The method we have described is a practical and sensitive tool for detailed analysis of sera with ambiguous serological findings. It allows reliable typing of donors for platelet alloantigens (eg, Pl^{A1} (Zw^{a}), Pl^{A2} (Zw^{b}), Bak^{a} (Lek^{a}), Pen) even with those sera that do not discriminate between negative and positive cells in the platelet immunofluorescence test due to HLA antibodies. Moreover, MAIPA will be a suitable method for platelet compatibility testing before platelet transfusion because it gives information about both HLA-specific (including noncytotoxic) and platelet-specific alloantibodies.

This assay takes advantage of the unique properties of MoAbs for diagnostic purposes. We are convinced that it will allow us to elucidate many of the unclear findings so often encountered in platelet serology.

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Serial Measurement of Lymphocytotoxic Antibody and Response to Nonmatched Platelet Transfusions in Alloimmunized Patients

By Edward J. Lee and Charles A. Schiffer

Serial evaluations of lymphocytotoxic antibody (LCTAb) and responsiveness to random donor platelet transfusion were reviewed in 234 patients who had developed LCTAb at some time during their treatment course. Seventy (30%) of these patients had significant falls in antibody levels. In 44 patients these declines occurred after further antigenic exposure was reduced either because no transfusions were administered or only histocompatible platelets were transfused. Forty patients with declines in LCTAb levels who were previously refractory to platelet transfusion were rechallenged with random donor platelets. Thirty-four of 35 clinically evaluable patients had good responses to these unmatched transfusions for 2 weeks to 36 months, and in 21 patients antibody did not return despite repeated transfusions. Thus, serial LCTAb measurements are helpful in the management of alloimmunized patients. Many patients will have decreases or a loss of LCTAb, either permanently or transiently, and can be successfully supported with more easily available unmatched random donor platelet transfusions.

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ALLOIMMUNIZATION represents one of the most challenging problems in the supportive care of patients with acute leukemia. Approximately 30% to 40% of patients with acute leukemia undergoing intensive chemotherapy will become alloimmunized to random donor platelet transfusions and require transfusion support from HLA-matched donors.[1,2] Few data exist on the long-term course of alloimmunized patients with respect to transfusion support or serological studies. Lymphocytotoxic antibody (LCTAb) is a serological marker that correlates with clinical alloimmunization.[3] In an earlier report, we described 114 patients in whom serial measurements of LCTAb were made.[1] Sixty-one of these patients never developed LCTAb and remained clinically responsive to random donor platelet transfusions. Forty-eight of these 114 patients (42%) developed LCTAb after their initial induction therapy. Of these 48 patients, eight patients had a significant fall in LCTAb activity at some time during their subsequent course. In this report, we expand this observation and demonstrate that a decline in LCTAb activity is more common than previously suspected. We also report that patients in whom LCTAb disappears can regain their responsiveness to random donor platelet transfusions and thus may be supportable for a period of time with more easily available standard platelet products. This in turn allows conservation of important resources such as HLA-matched donors[4] or autologous frozen platelet concentrates (AFPC)[5] and more safely allows delivery of intensive post-remission or reinduction chemotherapy.

MATERIALS AND METHODS

Patients. The records of patients who received serial platelet transfusions during treatment at the University of Maryland Cancer Center between the years of 1975 and 1986 who survived greater than 4 weeks were reviewed. Only patients who survived >4 weeks were considered to permit an adequate duration of time for patients to mount an antibody response. Of a total of 579 evaluable patients, 397 had acute nonlymphocytic leukemia (ANLL), 68 had acute lymphocytic leukemia (ALL), 43 had chronic myelogenous leukemia (CML), and 71 had other malignant diseases (Table 1). LCTAb levels were measured on admission for all patients with leukemia and serially thereafter. Nonleukemic patients had LCTAb activity measured at times at which they developed clinical problems suggesting that they might be alloimmunized. Thus, this nonleukemic population is skewed and overrepresents patients who had poor responses to platelet transfusions.

Patients were considered part of the study population if they had two consecutive specimens >20% positive (see the next section) followed by two consecutive measurements of LCTAb that were <20% positive at some time during their course. All such patients were then evaluated for the presence of clinical refractoriness.

LCTAb. LCTAb studies were performed by Dr Paul Terasaki, Los Angeles, through 1982 and thereafter by the Rh Typing Laboratory, Baltimore. A positive LCTAb is defined as cytotoxicity against >20% of the wells in a standard lymphocytotoxicity assay as described previously.[3,6]

Criteria for alloimmunization. The corrected count increment (CCI) was calculated according to the following formula: CCI = (posttransfusion platelet count − pretransfusion platelet count) × body surface area (m^2)/no. of platelets transfused ($\times 10^{11}$). Alloimmunization was defined clinically as a CCI of <7,500 one hour after platelet transfusion in the absence of any alternative explanations for a poor response such as splenomegaly, coagulopathy, fever, infection, or active bleeding[7] followed by a good response (one hour posttransfusion CCI >10,000) to an HLA-matched platelet transfusion.

RESULTS

Of the 397 patients with ANLL, 174 (44%) developed LCTAb at some time during their course. Twelve of 68 patients with ALL (18%), 17 of 43 patients with CML (40%), and 31 of 71 patients (44%) with other malignancies who had poor responses to platelet transfusions developed LCTAb. It should be emphasized that the overall rate of alloimmunization in nonleukemic patients was considerably <44% in that these patients were tested for LCTAb only if poor responses to platelet transfusions were observed. The patients with ANLL were significantly more likely to

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Table 1. Patient Characteristics

	n	Positive LCTAb
Total patients	579	234 (40%)
ANLL	397	174 (44%)*
ALL	68	12 (18%)*
CML, blast crisis	43	17 (40%)
Others	71	31 (44%)

*$P = .00002$, Fisher exact probability test.

develop LCTAb ($P = .00002$, Fisher exact probability test) than patients with ALL. Overall, 234 of 579 patients (40%) developed significant levels of LCTAb (Table 1).

Seventy of these 234 (30%) patients had decreases in the activity of LCTAb to <20%, whereas in 70% the LCTAb activity remained high. These reductions occurred in 44 patients after a period of time during which either no transfusions were administered or HLA-matched and/or AFPC were given and in 26 patients despite continued transfusion with random donor platelets.

Of these 70 patients, 12 had never been refractory to unmatched platelets and continued to respond to random donor platelet transfusions (Table 2). Notably, of these 12 patients, nine had only transient elevations of LCTAb levels during one period of supportive care. Eighteen of the remaining 58 patients did not receive unmatched platelet transfusions after a decline in LCTAb levels was noted either because no further transfusions were given (12 patients) or only HLA-matched or autologous platelet transfusions were given (six patients).

Five of the other 40 patients were rechallenged with random donor platelet transfusions, but at a time when complicating clinical factors such as splenomegaly, septicemia with hypotension, or disseminated intravascular coagulation were present. The poor responses of these five patients to random donor platelet transfusions were therefore uninterpretable.

One of the 35 evaluable refractory patients who was rechallenged had low increments after a transfusion of random donor platelets. This patient had a rapid, anamnestic rise in LCTAb levels. Thirty-four of the remaining 35 patients received between one and 83 (median, five) random donor platelet transfusions with good posttransfusion platelet increments. Six patients received more than 20 such transfusions. Thirteen of these 34 patients responded to nonmatched platelet transfusions for 2 weeks to 9 months, but 12 of the 13 thereafter again became refractory, which was associated with increases in LCTAb levels. In contrast, 21 patients never became refractory again despite repeated transfusions for up to 3 years, and only three developed subsequent increases in LCTAb activity. In ten of these patients, responsiveness could be evaluated for only 2 weeks because five of these patients died within 2 weeks and five did not require further platelet transfusion.

Table 2. Outcome in 70 Patients With Decreases in LCTAb Activity to <20%

70 patients with decreasing LCTAb activity
  12 never refractory
  58 refractory
    18 not rechallenged
    40 received further platelet transfusions
      34 good response to random-donor platelet transfusions
        21 never again refractory
          10 patients supported for up to 2 wk
          11 patients supported for 3 mos to 3 yr
        13 became refractory 2 wk to 9 mo later
      6 no response
        5 clinically unstable, no increase in LCTAb activity
        1 evaluable for response, rapid increase in LCTAb activity

DISCUSSION

LCTAb, which in most although not all patients was associated with refractoriness to random-donor platelet transfusion, developed at some time during the life of 40% of this large series of patients with acute leukemia. Of note is that the incidence of alloimmunization was significantly lower in patients with ALL than ANLL ($P = .00002$, Fisher exact test). This may reflect a difference in the disease process itself but more likely represents an effect of high-dose corticosteroids routinely used in ALL patients during initial therapy and antigenic exposure.

Serial evaluations revealed considerable fluctuation in antibody positivity. Importantly, random-donor platelet transfusions were effective in 34 of 35 previously refractory patients in whom decreases in LCTAb activity were detected. These responses to random-donor platelet transfusions suggest that serial measurements of LCTAb levels should be done in alloimmunized patients and that a decrease in LCTAb activity can be used to select patients who will respond well to random-donor platelet transfusion. It is striking that some patients could be supported for more than a year with random-donor platelet transfusions after having been alloimmunized and that some patients never again become refractory.

Other literature, particularly that describing patients with chronic renal insufficiency, has also described fluctuations in LCTAb.[2,8] Declines occurred predominantly in patients with only moderate levels of antibody activity. In contrast, in many of our patients high levels of LCTAb disappeared. Thus, 16 of the 34 patients who subsequently responded to random-donor platelet transfusions after a decline in LCTAb activity had been profoundly alloimmunized as documented by CCIs of ≤1,000.

The mechanism by which this decline in LCTAb activity occurs is unclear. In some patients, the decline followed a period in which no transfusions were given. With the lack of immunologic stimulus, it is not surprising that the levels of antibody would fall. In an analogous fashion, patients supported solely with either autologous or closely HLA matched platelet transfusions might be expected to have reductions in LCTAb levels. It is more striking, however, that 26 patients developed the reduction in LCTAb activity while they were actively receiving random-donor platelets. There were no common events that occurred in these patients such as the use of a particular chemotherapeutic approach or the presence or absence of a severe infectious process or complication. In these patients, neither the decrease of LCTAb activity nor the improved response to random-donor platelet transfusions was expected.

The provision of histocompatible platelet transfusions is a time-consuming and expensive undertaking. These data document the value of serial measurements of LCTAb levels in alloimmunized patients with leukemia who are undergoing chemotherapy so as to identify patients in whom random-donor platelet transfusions may be useful. Thus, it may be possible to conserve resources such as AFPC and HLA-matched, single-donor platelet transfusions for particular patients who are difficult to support with platelets. This may in turn extend the duration of time that a given patient may be effectively supported and more safely permit aggressive chemotherapy.

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Tumor Necrosis Factor: Receptors on Hematopoietic Cells

By Reinhold Munker, John DiPersio, and H. Phillip Koeffler

Tumor necrosis factor (TNF) is a monokine that is cytotoxic/cytostatic for a variety of tumor cells and has multiple effects on normal cells. We demonstrate that normal and malignant human myeloid cells express a single class of high-affinity receptors (400 to 1,900 per cell, K_D 20 to 90 pmol/L) for TNF. Mitogen-stimulated lymphocytes have a similar number of TNF receptors, whereas resting lymphoid cells have fewer receptors. RBCs and platelets have no detectable TNF receptors. No correlation is observed between the receptor number, receptor affinity, and the cytoxic effect of TNF on myeloid cell lines. Significant cytotoxic effects of TNF on the most sensitive myeloid cell line (HL-60 promyelocytes) could be seen at concentrations tenfold lower than those concentrations at which one half of the TNF receptors were occupied. Our data show that significant biologic effects of TNF can occur at low levels of receptor occupancy and resistance to TNF is not related to the absence of TNF receptors on certain myeloid leukemic cell lines.

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INTEREST IN THE BIOLOGIC EFFECTS of tumor necrosis factor (TNF) has increased since the gene was cloned and the recombinant protein has become available. Besides being cytotoxic or cytostatic for certain tumor cells, TNF exerts multiple effects on normal cells.[1-3] We and others recently studied the action of recombinant TNF on normal and malignant myeloid cells and found that TNF inhibited clonal growth of most myeloid leukemia cells, whereas normal myeloid progenitors (CFU-GM) were relatively less sensitive to the cytostatic effects of TNF.[4,5] In the present study we successfully iodinated TNF to high specific activity with full preservation of biologic activity. Iodinated TNF, after establishment of equilibrium binding conditions, was used to correlate TNF receptor number and affinity with its cytostatic effect on both sensitive and resistant myeloid leukemic cell lines.

MATERIALS AND METHODS

Recombinant, highly purified (≥99%) human TNF (generous gift of M. Shepard and B. Aggarwal, Genentech, Inc, San Francisco) had an activity of 3.8×10^7 U/mg when assayed on L-929 fibroblasts in the presence of 1 μg/mL of actinomycin. TNF was iodinated by the Iodogen method as described.[6] Briefly, 10 μg of purified recombinant protein was incubated for ten minutes at 4°C in an Iodogen (Pierce Chemical Co, Rockford, IL) coated glass tube with 1 mCi of ^{125}I-NA (Amersham Corp, Arlington Heights, IL). Free iodide was separated from TNF on a Sephadex G-25 column. An aliquot of the iodinated protein (~10^5 cpm) was run on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under reducing and nonreducing conditions, and autoradiography was performed according to standard procedures.[7] For the binding assays, 8×10^5 cells typically were incubated with dilutions of iodinated TNF (5×10^3 to 2×10^6 cpm) in a total volume of 220 μL for 90 minutes at 24°C. The binding medium was α culture medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), 1% penicillin-streptomycin (Irvine Scientific) and 20 mmol/L HEPES (Sigma Chemical Co, St Louis). The incubation was performed in Flacon microtest multiwell plates (Becton Dickinson, Oxnard, CA). After the incubation the cells were washed three times in Eppendorf tubes with binding medium. To decrease binding to plastic, the tubes used for washing the cells were changed twice. Finally, the cell-associated radioactivity was determined with a Beckman Gamma 300 counter (Beckman Instruments Fullerton, CA). To determine nonspecific binding, the cells were also incubated in the presence of a 100-fold excess of unlabeled TNF. The specific activity of ^{125}I-TNF was determined by self-displacement analyses in which KG-1A cells were incubated with 0.4 to 10 ng unlabeled TNF in the presence of 4×10^4 to 5×10^5 cpm ^{125}I-TNF.[8] After establishing equilibrium binding conditions (24°C, two hours), TNF receptor number and affinity expressed on a variety of normal and leukemic cell types were determined by the method of Scatchard.[9]

Mononuclear cells from bone marrow and peripheral blood were isolated from normal and leukemic donors on Ficoll gradients. Informed consent was obtained from each donor. Leukemic cells isolated from peripheral blood or bone marrow contained ≥90% malignant cells as determined by morphological examination of Giesma-stained cytocentrifuged preparations. Bone marrow mononuclear cells were adhered to plastic for >3 hours to remove macrophages; the final cell mixture contained about 65% early myeloid cells (myelocytes and less mature granulocyte precursors) and 30% erythroid percursors. Normal granulocytes were obtained after dextran sedimentation for 90 minutes, Ficoll sedimentation, and hypotonic lysis of the remaining red cells. Thymocytes were isolated from thymus specimens from children undergoing cardiac surgery.

Myeloid cell lines were cultured as described.[10] For binding studies, the cells obtained during exponential growth phase were washed twice in tissue culture medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin.

RESULTS

Under reducing conditions, iodinated recombinant TNF migrated as a single band on SDS-PAGE with an approximate molecular weight (mol wt) of 17,000 (Fig 1). Under nonreducing conditions an additional faint band at approxi-

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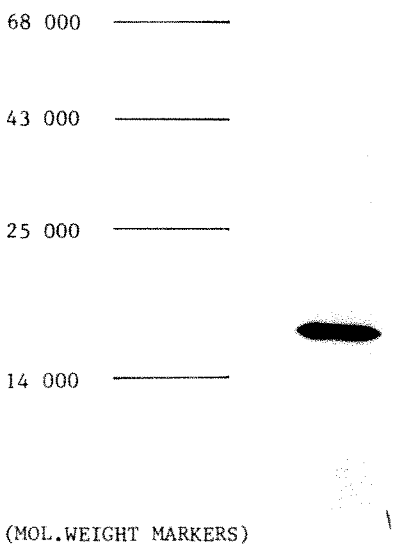

Fig 1. Autoradiogram of ^{125}I-TNF separated by 12% SDS-PAGE under reducing conditions. On the left, the position of mol wt markers is indicated.

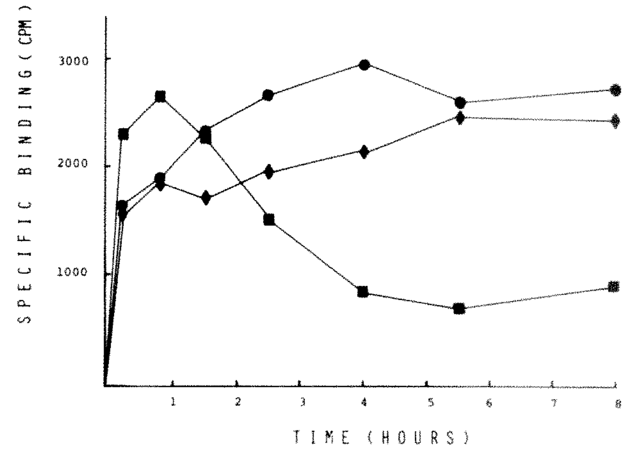

Fig 3. Time and temperature dependence of the specific binding of ^{125}I-TNF to the myeloid cell line HL-60. The binding experiments were performed at 0°C (♦), 23°C (●), and 37°C (■). All values are corrected for nonspecific binding obtained at a 100-fold excess of unlabeled TNF.

mately 34,000 was observed. Iodinated TNF retained its biologic activity (>85%) measured by the inhibition of colony formation of HL-60 cells (data not shown) and was stable for about 2 months. By using the myeloid leukemia cell line KG-1A, the specific activity of ^{125}I-TNF was determined by self-displacement (Fig 2) and was found to be approximately 4.2 × 10^5 cpm/ng (7.1 × 10^6 cpm/pmol/L, 3,227 Ci/mmol/L TNF). The binding of TNF to myeloid cell lines occurred rapidly (Fig 3). At 0°, 23°, and 37°C approximately 60% to 90% of the maximal binding occurred by 15 minutes of incubation. At 37°C maximum specific binding was reached at 45 minutes, with a decline over the next seven hours, presumably due to intracellular uptake and degradation. At 23°C and 4°C the peak of specific binding was reached more slowly, with a plateau around 1.5 to five hours. All subsequent experiments were performed at 23°C with a 90-minute incubation. Depending on the cell type, 70% to 95% of the binding was to a single class of high-affinity, specific receptors. Equilibrium binding data for the myeloid cell line KG-1 analyzed by the method of Scatchard[9] are shown in Fig 4.

The number and affinities of TNF receptors expressed on a variety of normal cell types, myeloid cell lines, and fresh leukemic blasts from patients with various leukemias are shown in Table 1. We found that all hematopoietic cells except RBCs and platelets expressed a single class of high-affinity receptors for TNF with a dissociation constant ([K_D] ~ 43 pmol/L). Granulocytes have about three times as many receptors (450 receptors/cell) than peripheral blood

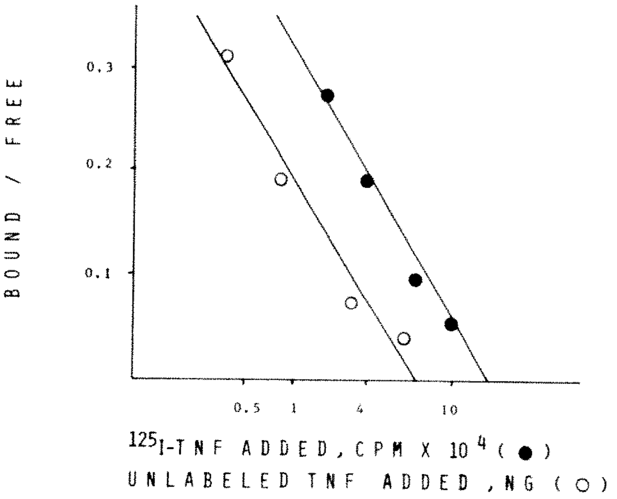

Fig 2. Determination of specific activity of ^{125}I-labeled TNF by self-displacement analysis. The bound/free ratios are plotted against the total radioactivity (0.5 × 10^4 to 10^5 cpm) added (●) and against the amount of unlabeled TNF added (0.5 to 10 ng) (○). A total of 8 × 10^5 KG-1A cells was used.

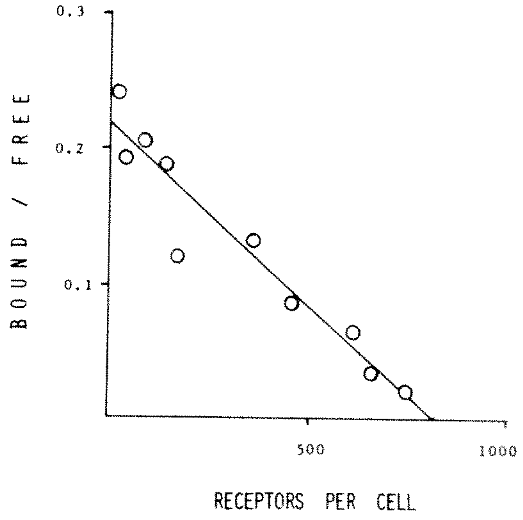

Fig 4. Scatchard plot for the myeloid cell line KG-1. Each circle represents the mean of triplicate data points.

Table 1. TNF: Number of Binding Sites per Hematopoietic Cell, K$_D$, and Growth-Inhibitory Concentration

Cell Type	n	TNF Receptors/Cell	K$_D$ (pmol/L)	ED$_{50}$ of Clonal Inhibition (pmol/L)
Normal cells				
Granulocytes	2	444 ± 95	29 ± 5	
Peripheral blood lymphocytes	3	141 ± 53	18 ± 1	
PHA-activated blood lymphocytes	1	413	73	
Bone marrow mononuclear cells	2	326 ± 41	36 ± 12	
Thymocytes	2	94 ± 0	23 ± 1	
RBCs	2	0	0	
Pooled platelets	1	0	0	
Myeloid cell lines				
HL-60 (promyelocytes)	3	776 ± 39	42 ± 6	4
EM-3 (myeloblasts)	1	1,167	48	5
U937 (monoblasts)	1	550	22	8
KG-1 (myeloblast)	2	676 ± 140	31 ± 5	29
KG-1A (undifferentiated myeloblast)	1	598	27	97
HEL (erythromyeloblast)	1	517	37	>200
K562 (erythromyeloblast)	1	537	28	>200
THP1 (monoblast)	1	1,870	94	>200
Leukemia cells from patients				
AML (H.A.)	1	363	29	
AML (C.O.)	1	893	89	
B-CLL (J.A.)	1	567	28	
B-CLL (T.E.)	1	22	37	

Abbreviation: PHA, phytohemagglutinin.

Equilibrium saturation binding curves were determined on the cells as described in both Material and Methods and Fig 4. Scatchard plots were made, and the K$_D$ (concentration that gave half-maximal binding) and the number of binding sites per cell was obtained from the x-intercept. The ED$_{50}$ is the concentration of TNF required to inhibit half the cells using the conditions of our standard clonogenic assay, and the ED$_{50}$ results are from Munker and Koeffler.[5] Results represent means and SD; n represents the number of experiments. Each experiment was carried out in triplicate.

lymphocytes (150 receptors/cell). Mitogen-stimulated peripheral blood lymphocytes expressed about three times more TNF receptors than unstimulated lymphocytes. All the myeloid leukemia cell lines had receptors for TNF (range, 520 to 1,870 receptors/cell). No correlation was noted between the number or affinity of TNF receptors expressed per cell and the in vitro cytostatic effect of TNF. For example, sensitive myeloid cell lines (HL-60, EM-3, U937) expressed fewer receptors than the relatively resistant cell line THP-1 (Table 1). The K$_D$ for the TNF receptors ranged between 19 to 95 pmol/L on those cells that expressed TNF receptors and was not significantly different on sensitive and resistant myeloid cell lines.

Of interest was that the concentrations of TNF required to inhibit 50% of the in vitro clonal growth of the HL-60, EM-3, and U-937 myeloid cell lines was three- to tenfold lower than the K$_D$s obtained by equilibrium binding.

We also examined leukemic cells from two patients with acute myelogenous leukemia (AML) and two patients with chronic lymphocytic leukemia (CLL) of the B lymphocyte cell type for their expression of TNF receptors. The leukemic cells from the AML patients had numbers of TNF receptors comparable to those found on myeloid leukemia cell lines. Cells from the two cases of CLL had heterogeneous binding of ^{125}I-TNF, with cells of one patient (J.A.) having 510 TNF receptors per cell and the other having 22 TNF receptors per cell. The leukemic cells from all four patients expressed a single class of high-affinity TNF receptors (K$_D$, 30 to 90 pmol/L).

DISCUSSION

Recently, the receptors for a variety of lymphokines and monokines including interferons (INF),[11,12] interleukin 1,[13] colony-stimulting factors,[14,15] and TNF[6,16-20] have been preliminarily characterized. These receptors are involved in the stimulation and downregulation of cellular growth processes.[21] Receptors for TNF were demonstrated on fibroblasts,[16,17] endothelial cells, adipocytes, myocytes, liver membranes,[18,19] granulocytes,[22,23] and several tumor cell lines.[6,20] These cell types expressed approximately 1,000 to 5,000 TNF receptors with K$_D$s between 3 and 600 pmol/L.[16,24]

We demonstrate that normal human hematopoietic cells, malignant myeloid and lymphoid cells, and myeloid leukemic cell lines have receptors for TNF. The binding of TNF to all the cells is extremely rapid, specific, and reversible. All cell types express a single class of high-affinity TNF binding sites. The affinity of binding of TNF did not vary greatly between the cell types. The decrease in specific binding after ≥1.5 hours that we observed at 37°C is consistent with the internalization and intracellular degradation of ^{125}I-TNF as previously shown by others.[16,17,24] We found that the numbers of receptors expressed on normal myeloid cells (300 to 500/cell) and on myeloid leukemia cells (500 to 1,800/cell) were higher than on peripheral blood lymphocytes and thymocytes (90 to 140 receptors/cell). Also, activated lymphocytes express about threefold more TNF receptors than resting lymphocytes or thymocytes (approximately 400 *v* 100 receptors, respectively). The mechanisms involved in the increased expression of TNF receptors on activated lymphoid cells are unclear but may include local membrane effects of the stimulating agents used (eg, PHA and 12-0-tetradecanoylphorbol-13-acetate) or through the production of INFs. INFs have been shown by others to both upregulate the number of TNF receptors and to enhance the biologic effects of TNF on responsive cell types in vitro.[21,22,25,26] Platelets and RBCs do not express detectable TNF receptors. The affinity constants determined for the TNF receptors expressed by the hematopoietic cells described here were comparable (20 to 90 pmol/L) to those reported for TNF receptors expressed by a variety of nonhematopoietic cells.[6,16]

The expression of TNF receptors on hematopoietic cells suggests that TNF may have an action on these cells. Studies have shown that aggregation, superoxide generation, phagocytosis, and polarization of neutrophils can be enhanced by TNF.[22,23] TNF at very low concentrations has a cytotoxic or cytostatic effect on normal activated lymphocytes[27] and myeloid leukemia cells.[4,5] In addition, TNF has been shown to enhance the killing of schistosoma by mature eosinophils.[28,29]

We found no correlation between the number of TNF receptors expressed on the myeloid leukemic cell lines and the biologic sensitivity of these cell lines to the growth-inhibitory effects of TNF. Several lines including HEL, K562, and THP-1 were resistant to TNF but expressed comparable numbers of receptors when compared with the numbers expressed by the sensitive lines presented here. Similar findings have been reported for several other tumor lines.[20] We have also found that several small cell lung cancer cell lines do not express detectable TNF receptors and are totally resistant to the cytotoxic/cytostatic effects of TNF.[30] Taken together, these results suggest that expression of TNF receptors is necessary but not sufficient for the biologic effect of TNF. In addition, we found no correlation between the ED_{50} of clonal inhibition mediated by TNF and the K_D determined for the TNF receptor on the sensitive myeloid leukemic cell lines HL-60, KG-1, KG-1a, EM-3, and U937. Furthermore, our studies suggest that in the three most sensitive myeloid leukemic cell lines (HL-60, EM-3, U937), the biologic and cytostatic effects of TNF occur at concentrations of TNF where only a few of the TNF receptors could be occupied. For example, the concentration of TNF needed to inhibit the clonal growth of HL-60 cells by 50% (4 pmol/L) was tenfold lower than that required to saturate 50% of the TNF receptors (42 pmol/L) on these same HL-60 cells. Silberstein et al[28,29] have recently shown that the concentration of TNF required to induce 50% maximal killing of schistosoma by normal human eosinophils was approximately 1×10^{-13} mol/L. This is approximately 1,000-fold lower than that concentrated required to saturate 50% of the TNF receptors on these eosinophils. These results would imply that significant biologic effects can occur at extremely low levels of receptor occupancy or that a separate higher-affinity TNF receptor exists on HL-60 cells that could not be detected by conventional equilibrium binding studies presented here. Peetre et al[24] has suggested the presence of such higher-affinity TNF receptors on several human myeloid leukemic cell lines. In addition, the extreme sensitivity of myeloid leukemic cell lines to TNF may also result from the production of synergistic factors such as γ- and β_2-INF or TNF itself. Both γ- and β_2-IFN have been shown to sensitize cells to the effects of TNF.[20,23,24,28,29,31] This enhancement could not be totally explained by a change of TNF receptor affinity or by the slight increase in the receptors expressed after γ-IFN treatment of myeloid cells.[31]

During the time we were completing our studies, several reports also showed that myeloid leukemic cells have TNF receptors.[24,31] Scheurich et al found that HL-60, U937, and K562 expressed TNF receptors, whereas a K562 variant (K562 C1) did not have detectable receptors.[31] Preliminary characterization of the TNF receptor by chemical cross-linking studies by these investigators demonstrated that myeloid cells expressed a single TNF receptor protein with an apparent mol wt of 76,000 daltons. Similar cross-linking studies using murine fibroblasts yielded two specific bands, which implied mol wts of 95 kd and 75 kd for the TNF receptor.[16] Our binding studies were done at 23°C. Cells from myeloid leukemia lines had about 550 to 1,900 TNF receptors per cell with K_D of 20 to 95 pmol/L. In contrast, Peetre et al[24] and Scheurich et al[31] found at 0°C that cells from myeloid leukemia lines had approximately 1,500 to 3,000 TNF receptors per cell with K_D of 150 to 300 pmol/L. Our finding of fewer TNF receptors with a higher affinity on the same myeloid cell lines might have occurred because of a difference in either reagents, the temperature at which the binding studies were performed, or possibly a difference in the cells. Nevertheless, each investigative group found that TNF was able to exert cytostatic effects on the TNF-sensitive myeloid cells at concentrations of TNF at which very low levels of receptor occupancy exists.

Myeloid cells can be induced to synthesize TNF[32,33]; at the same time these cells express receptors for TNF. Further studies are required to determine whether TNF can have a biologic effect on the myeloid cells that synthesize TNF under normal and abnormal circumstances, which would suggest a type of autocrine regulation. Recently, Cannistra et al[34] have shown that U937 cells incubated with GM-CSF rapidly die in culture, probably due to the induction of TNF synthesis by these cells. In addition, a better understanding of postreceptor events involved in transmembrane signal tranduction may help explain the more lethal effects of TNF on leukemic compared with normal myeloid cells and how TNF can exert profound biologic action at low levels of receptor occupancy.

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Purification of Human Megakaryocytes by Fluorescence-Activated Cell Sorting

By Aaron Tomer, Laurence A. Harker, and Samuel A. Burstein

For direct studies of growth control, a method was developed to purify viable human megakaryocytes to homogeneity from routine normal bone marrow aspirates. An initial separation of marrow over a 1.050 g/mL Percoll density cut was used to enrich megakaryocytes. After washing, the cells were specifically labeled with a fluoresceinated monoclonal antibody or F(ab')$_2$ fragment to the platelet glycoprotein (GP) IIb/IIIa complex. Megakaryocytes were selectively sorted by using Becton Dickinson FACStar flow cytometer on the basis of a fluorescence intensity >50-fold that of control cells. To increase resolution and purity the sorting rate was adjusted to one cell in 13 formed drops, and negative events that coincided with positive ones were aborted. Two thirds of the isolated cells were large, morphologically recognizable megakaryocytes with a forward light scatter fourfold that of the main cell population. Microscopic examination showed these cells to be ≥98% megakaryocytes with a diameter of 20 to 46 µm and a ploidy range of 2N to 64N with a mode of 16N. The small highly fluorescent cells were 10 to 21 µm in diameter, and their ploidy range from 2N to 32N with main ploidy classes of 2N and 4N. The majority of these small cells also positively reacted with monoclonal antibody to platelet GPIb. The isolated cells were cultured in either Iscove's or leucine, lysine-deficient RPMI 1640 medium with 10% human plasma. The cells were maintained in culture more than three days and were capable of synthesis of both DNA and protein as assessed by radiolabeled thymidine and amino acid incorporation. Moreover, the isolated megakaryocytes were capable of responding to recombinant granulocyte-macrophage colony-stimulating factor. The data show that human megakaryocytes can be purified from routine marrow aspirates on the basis of a lineage marker and that they are capable of growth in vitro.

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STUDY OF THE BIOLOGY of megakaryocytes has proved difficult, in part due to their rarity (constituting about 0.05% of all nucleated bone marrow cells), fragility, and their tendency to aggregate.[1-3] Thus, a large number of marrow cells and a highly selective method for megakaryocytic identification are needed for their isolation or analysis. Consequently, most studies of isolated megakaryocytes have used marrow flushed or scraped from the bones of experimental animals to achieve high cell yield with little admixture with blood and activated coagulation factors.[4-13] For studies of human megakaryocytes this source is limited to surgical amputations.[1,14-16]

Both human and animal megakaryocytes have been isolated with variable results by using velocity centrifugation, which separates cells based primarily on their size, and density gradient centrifugation.[1,3-5,14-17] Using marrow from human ribs Rabellino et al[1,14] and Sitar[16] obtained recoveries of 63% to 89% and purities of 68% to 94%. Notwithstanding these encouraging results, this method is limited by the need for ribs and the lack of specific megakaryocytic markers independent of size, which might result in a preferential selection of cells within the megakaryocytic population.[2,3,11]

Counterflow centrifugal elutriation (CCE) is an alternative approach. This procedure is based on the differences in sedimentation velocity between megakaryocytes and smaller cells.[2,7,8,18] Enrichment by CCE results in 60% to 70% recovery and about 25% purity, which may be increased up to 50% to 65% when combined with density centrifugation.[12,19] This method also inherently selects for larger megakaryocytes.

To overcome some of these technical limitations, flow cytometry has been introduced for the analysis of megakaryocytes.[5,6,9-11] This method has been shown to be sensitive and rapid for the analysis of infrequent cell populations, even in complex cell mixtures such as exists in bone marrow.[20] To prepare highly pure, viable human megakaryocytes isolated on the basis of a lineage marker on a routine basis, we have developed a method using fluorescence-activated cell sorting of normal bone marrow aspirates after labeling with a monoclonal antibody to the glycoprotein IIb/IIIa (GPIIb/IIIa) complex. In this report we describe the technique, its advantages and limitations, together with its applicability to studies of megakaryocytopoiesis in vitro.

MATERIALS AND METHODS

Marrow preparation. Six to 8 mL of bone marrow was collected from a single site (with one reposition of the needle) from the posterior iliac crest of normal volunteer donors. The marrow was aspirated after obtaining informed consent according to the guidelines of the Human Subjects Committee of the Research Institute of Scripps Clinic. Cells were collected into 10-mL plastic syringes containing $\frac{1}{10}$ vol acid-citrate-dextrose, formula A (ACD-A) EDTA, and prostaglandin E$_1$ (PGE$_1$; Sigma Chemical Co, St Louis) to a final concentration of 2.5 mmol/L and 2.2 µmol/L respectively. The marrow was gently pipetted, passed through a 200-µm monofilament nylon filter, and diluted with cold Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) containing 13.6 mmol/L sodium citrate, 2.2 µmol/L PGE$_1$, 1 mmol/L theophylline, 3% bovine serum albumin (BSA, fraction V; Calbiochem, La Jolla, CA), and 11 mmol/L glucose, and adjusted to a pH of 7.3 and an osmolarity of 290 mOsm/L. Hereafter, this supplemented PBS is designated MK medium.

In initial studies bone marrow was aspirated into heparin (40 U/mL, preservative-free)-containing syringes and diluted subse-

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quently with medium containing citrate, adenosine, and theophylline in Hank's buffer[4] (CATCH). This resulted in frequent cell aggregation in the Percoll gradient and cell clumping in the subsequent washing step. To reduce the likelihood of cellular aggregation, the heparin was replaced with ACD-A solution containing 2.5 mmol/L EDTA and 2.2 μmol/L PGE$_1$ (which was also used to rinse the aspiration needle). When 10 μmol/L of the synthetic thrombin inhibitor, D-phenylanalyl-L-propyl-L-arginine-L-chloromethylketone was added to the aspiration mixture, fibrin clot formation was reduced, but at the expense of increased cellular aggregation within the Percoll gradient. The dilution medium was supplemented with 3% BSA and initially with 2.5 mmol/L EDTA. This concentration of EDTA was subsequently found to be unnecessary, as was the addition of DNase. Indeed, it is unlikely that this divalent cation–requiring nuclease is active in citrate- and/or EDTA-containing buffers. To improve viability, the theophylline concentration was reduced from 2 mmol/L (the original concentration in CATCH medium) to 1 mmol/L, and the adenosine component of CATCH medium was omitted with no observable increase in aggregation tendency.

Cell count, viability, and size measurement. Marrow cells were enumerated with a Hematology Series 15 Cell Counter (Baker Instruments, Allentown, PA) or a hemocytometer. Viability was assessed by trypan blue exclusion. The diameters of megakaryocytes freshly isolated by cell sorting (see the following sections) were measured with an ocular micrometer as previously described.[21]

Fractionation of marrow cells. The cell suspension was adjusted to 20 × 10^6 cells/mL, mixed with Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to a final density of 1.020 g/mL (isotonic 83.1% Percoll diluted with PBS containing 13.6 mmol/L sodium citrate), layered over 12 mL Percoll of density 1.050 g/mL, and subsequently overlayered with 10 mL MK medium. After centrifugation at 400 g for 20 minutes at 15°C, the upper medium layer was removed, and the cells were collected from the upper Percoll layer and the interface ($\leq$1.050 g/mL). The collected cells were then washed by mixing with 1.5 vol of cold MK medium containing 6% BSA and centrifugated at 300 g for ten minutes at 15°C. The supernatant was decanted and the cell pellet was resuspended in the same medium and stored on ice for antibody labeling.

Adjustment of the cell concentration to no greater than 20 × 10^6/mL and admixture of the cells with Percoll to a final density of 1.020 g/mL (before layering over the 1.050 g/mL Percoll) was found to be helpful in preventing aggregate formation during centrifugation, presumably because the cells were dispersed in a larger volume rather than being concentrated at the interface. To prevent cell clumping during the successive washing step, the concentration of BSA was increased to 6%.

Megakaryocyte labeling. Megakaryocytes were directly labeled with saturating concentrations of fluoresceinated IgG1 mouse monoclonal antibodies (or F(ab')$_2$ fragment thereof) directed to the platelet GPIIb/IIIa complex. The antibodies reacted only with platelets and megakaryocytes.[21] These antibodies (designated P$_1$ and P$_4$) were a gift from Dr Z.M. Ruggeri of the Research Institute of Scripps Clinic and were fluoresceinated to an F/P ratio of 3:1 by standard techniques.[22] The cell count was adjusted to 10^7/mL with MK medium containing 6% BSA and then incubated with 8 μg/mL antibody for 30 minutes on ice. Dead cells as well as unbound antibody were removed by centrifugation at 100 g for seven minutes at 15°C over 10% BSA.[23] The cell pellet was then resuspended with MK medium containing 10 mmol/L HEPES buffer and 50 U/mL penicillin-streptomycin solution. The saturating concentration of the monoclonal antibodies was determined by quantitative flow cytometric measurements of the fluorescence of large megakaryocytes. An aliquot of the cell suspension was incubated under identical conditions with a fluoresceinated IgG1 mouse monoclonal antibody to human thyroglobulin and used as the control cells. The total time required to prepare the marrow cells for sorting was two to 2.5 hours.

Flow cytometry. Megakaryocytes were sorted with a FACStar flow cytometer (Becton Dickinson, Mountainview, CA) equipped with a 2 W Coherent, Innova 70 argon ion laser and a 120-μm-diameter nozzle. The sheath pressure was adjusted to the minimum level allowing stable drop formation (6.2 psi), with a sample pressure of 2 psi. The drop formation frequency was set to about 13.1 kHz, and the cell concentration was adjusted to allow sorting of no greater than 1,000 events/s. The number of drops collected for a single positive event was set to three to improve the yield without compromising the purity because only one of 13 drops contained detected particles. Purity was also enhanced by aborting negative events coinciding with positive ones. The stability of the fluid jet and the drop delay setting were monitored frequently. Small particles up to 7 μm were electronically gated out on the basis of forward-angle light scatter (FSC) by using 5.3-, 7.8-, and 10.2-μm standard polystyrene beads (Becton Dickinson), human RBCs, and peripheral blood lymphocytes. The laser emission was adjusted to deliver 200 mW at 488 nm for excitation.

A 570-nm dichroic mirror was used to separate the fluorescence emissions of the fluorescein (green) and the propidium iodide (red) when it was used for concurrent ploidy measurement (see the next section). The green fluorescence was further filtered by a 530/30-nm-band pass-filter, and red fluorescence was passed through a 625/35-nm-band pass-filter before detection by the photomultipliers.

Megakaryocytes were identified by their distinct high green fluorescence (>50-fold that of the main cell population or that of the control cells). Megakaryocytes formed a discrete subset when the fluorescence emission was plotted against FSC (either by dot plot or contour histogram). Small megakaryocytes were defined by FSC levels corresponding to cell diameters up to 20 μm. The levels of fluorescence intensity, FSC, and side scatter of each cell were collected by a Hewlett Packard 9144 computer, and the data were analyzed with a Consort 30 computer program (Becton Dickinson). When cells were collected for culture, the fluidic system was first sterilized with 70% ethanol followed by sterile PBS, and the cells were sorted into MK medium. The sample tubes were kept on ice and the collecting tubes were kept at 4°C. The adjustment of the flow cytometer required about one hour when experience was achieved. Sorting of an average cell sample required about two hours.

Cell ploidy determination. Cell ploidy was determined by flow cytometric measurement of the relative cell DNA content after staining by propidium iodide in hypotonic citrate solution as described by Krishan.[24]

Immunoenzymatic staining for GPIb. Small cells, isolated on the basis of high fluorescence level only (and thus high expression of the epitope identified by P$_1$ or P$_4$), were examined for the additional lineage marker GPIb. The isolated cells were cytocentrifuged onto poly-D-lysine–coated slides, dried, fixed with acetone-ethanol (3:17) and stored at −20°C. The following procedure was carried out in a dark, humid chamber at room temperature, using PBS for all washing steps. The slides were rehydrated for 20 minutes and incubated with 2% horse serum for 30 minutes. After washing, a 1:1,000 dilution in 0.15% BSA (crystalline grade, Sigma) of a mouse monoclonal antibody (a gift from Dr Z.M. Ruggeri) directed to GPIb was added for 120 minutes. Control samples were incubated with mouse monoclonal antibody to human thyroglobulin. The slides were then washed extensively and incubated with a 1:25 dilution of biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA) for 30 minutes. After washing, the slides were incubated with an avidin-biotin–alkaline phosphatase complex (Vectastain, ABC-AP; Vector) for 30 minutes and then with the enzyme substrate (kit I) for 20 minutes, washed with water, and counterstained with hematoxylin.

Megakaryocyte culture. Isolated marrow megakaryocytes were concentrated by centrifugation at 100 g for five minutes at 15°C and resuspended in 120 μL of MK medium. A diluted cell sample was counted, and the viability was assessed by trypan blue dye exclusion. Five hundred to 3,000 viable megakaryocytes were plated in duplicate microculture wells (Corning Glass Works, Corning, NY) containing 200 μL Iscove's medium (Irvine Scientific, Irvine, CA) for studies of thymidine incorporation or leucine, lysine-deficient RPMI 1640 medium (Irvine) for studies of amino acid incorporation. Both media were supplemented with 10% dialyzed normal human plasma, 200 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol. In some experiments the serum was replaced with 1% Nutricyte (J. Brooks Laboratories, San Diego) and 3% crystalline BSA (Sigma).

In subsequent experiments, cells were collected directly into tissue culture chambers mounted on plastic slides (Lab-Tek, Miles Scientific, Naperville, IL) containing culture medium. Cultures were incubated in a humidified 5% CO_2 tissue culture incubator at 37°C.

Radioisotope incorporation. To investigate the capacity of megakaryocytes to synthesize DNA and protein, cultures were labeled with 2 μCi of either ^{3}H-thymidine (^{3}HTdR, 87 Ci/mmol) or a ^{3}H-leucine-lysine mixture (120 Ci/mmol and 77 Ci/mmol respectively; Amersham Corp, Arlington Heights, IL). Similar amounts of radioisotopes were added to control wells containing no cells. After 20 hours in culture, the cells were harvested into 10% cold trichloroacetic acid (TCA). TCA-precipitable material was collected on 2.4-cm-diameter GF/C glass microfiber filters (Whatman, Maidstone, England), washed with 10% TCA, and counted with a liquid scintillation counter (model LS 7000, Beckman Instruments, Fullerton, CA). In some experiments aminopterin and hypoxanthine (4×10^{-7} mol/L and 1×10^{-4} mol/L final concentrations, respectively) were added to the culture medium to enhance the incorporation of thymidine. The significance of differences between incorporation rates was assessed with Student's *t* test.

Granulocyte-macrophage colony-stimulating factor (GM-CSF). To assess the response of isolated megakaryocytes to hematopoietic growth factors, sorted megakaryocytes were cultured in the presence of human recombinant GM-CSF, a gift from Dr Ken Kaushansky, University of Washington, Seattle. The factor was high-performance liquid chromatography–purified from serum-free medium conditioned by COS monkey kidney cells tranfected with human GM-CSF cDNA. The final preparation was about 70% pure, and its specific activity as assayed on human bone marrow was approximately 1×10^4 U/mL. The preparation was diluted to 25 U/mL with 2% human platelet-poor plasma in PBS.

RESULTS

Cell separation. As shown in Table 1, the yield of nucleated bone marrow cells from 6 to 8 mL of aspirated marrow was $434 \pm 280 \times 10^6$ (n = 18). It was estimated that morphologically identifiable megakaryocytes constituted about 0.05% of this cell fraction.[1-3,21] Percoll density gradient fractionation resulted in the depletion of 97.5% ± 0.5% of the total marrow cells and an increase in megakaryocytic frequency to 2.1 ± 0.4, which represents a 40-fold enrichment. The light-density ($\leq$1.050 g/mL) fraction contained >90% of morphologically recognizable megakaryocytes.[1,3,14,21] After the labeling procedure with the antibody and two washing steps, the yield of the cells initially recovered from the light-density fraction was 50% (the yield of cells from the dense Percoll fraction >1.050 g/mL was 70%). Cell viability before sorting was about 90%.

Selection of GPIIb/IIIa-positive cells. Figure 1 demonstrates the fluorescence distribution of megakaryocyte-enriched bone marrow labeled with the GPIIb/IIIa-specific monoclonal antibody (A, the intact antibody; B, the $F(ab')_2$ fragment) compared with the same sample labeled with the control monoclonal antibody to human thyroglobulin (C). A peak of highly fluorescent cells was detected in the sample labeled with the specific probe. These cells constitute about 2.5% of the total cell population (similar to the percentage of megakaryocytes estimated on this cell preparation using morphologic criteria) and exhibit a fluorescence intensity of 50- to 180-fold that of the major cell population (the higher level being observed with the $F(ab')_2$ fragment, B).

To improve megakaryocytic identification and to set criteria for reproducible selection, the fluorescence intensity was plotted against FSC, a parameter reflecting, in large part, cell size (Fig 2).[20] The bidimensional plot revealed a discrete subset with both high fluorescence intensity and an FSC fourfold that of the major cell population (Fig 2, upper right quadrant). After sorting and staining with hematoxylin, these cells appeared to be morphologically pure megakaryocytes (Fig 3). Sorting and staining of the unselected marrow cell population (Fig 2, lower quadrants) revealed that the number of megakaryocytes in this fraction was between 2.5% and 5% of that found in the selected population.

The yield of megakaryocytes from an average marrow

Table 1. Isolation of Megakaryocytes

	Nucleated Cells		Megakaryocytes			
Fraction	Number (× 10^6)	Percentage of Total	Percentage of Nucleated Cells	No. (× 10^3)	Percentage of Total	Viability (%)
Unfractionated marrow	154-714	100	0.04-0.06*	173-260†	100	>90
Percoll density cut (<1.050 g/mL)	3.9-17.9	2.5	1.7-2.5	184-260	90	
Presorting‡	2.0-9.8	1.25	1.7-2.5	92-130	45	90
Postsorting	0.074-0.104	0.02	98-100	72-102§	36	
				36-51‖	18	50

*An estimate based on previous work.[1-3,21]

†The calculation is based on the mean of the nucleated cell number and the frequency of megakaryocytes in the preparation (n = 18).

‡Cell suspension after labeling with monoclonal antibody and two washing steps.

§Cells collected directly into chamber slides containing medium.

‖Cells colected into tubes and concentrated by centrifugation.

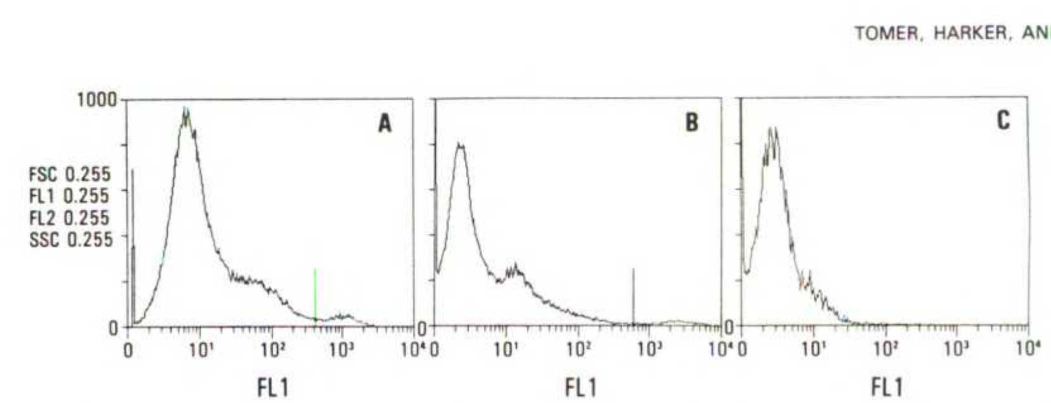

Fig 1. Fluorescence distribution of megakaryocyte-enriched marrow cells labeled with P_1 (A), the $F(ab')_2$ fragment (B), or antihuman thyroglobulin (C). The abscissa represents the relative fluorescence intensity and the ordinate cell number per channel. The numbers to the right of the flow cytometric parameters show the channel ranges that are opened, which indicates that the presented populations are unselected. The vertical lines (in A and B) indicate the gate position for selection of the highly fluorescent subset. Each curve represents an analysis of 50,000 cells.

aspirate of 4 × 10^8 cells was about 40,000 (Table 1) and was dependent upon the efficiency of sorting and manipulation of the collected megakaryocytes. The efficiency of sorting at optimal settings as defined by either resorting of a collected sample or comparing the electronically sorted events with the actual number of cells collected directly on slides was 80% to 90%. Concentrating the purified megakaryocytes in the collecting tube by centrifugation resulted in about a 50% loss. Subsequently, megakaryocytes were sorted directly into plastic chambers containing medium. This modification enhanced the yield up to 80,000 cells.

Sorting of the entire highly fluorescent cell population (Fig 2, horizontal solid line) resulted in inclusion of small cells which constituted 20% to 30% of the total selected cells. Only one third of these cells were morphologically recognizable megakaryocytes based on cell size and nuclear lobulation. To define further their state of differentiation, these cells were stained immunocytochemically with a monoclonal antibody to GPIb. Of 1,000 cells examined, 70% to 80% reacted positively.

Differentiation between small and large megakaryocytes was based on FSC (which was then translated to an electronic gate). By using standard beads, RBCs, and peripheral blood lymphocytes, small cells were defined as cells exhibiting FSC up to the level corresponding to a 20-μm diameter. About 97% to 98% of the total marrow cell population was included in this size range (vertical solid line in Fig 2). The size of the small megakaryocytes was

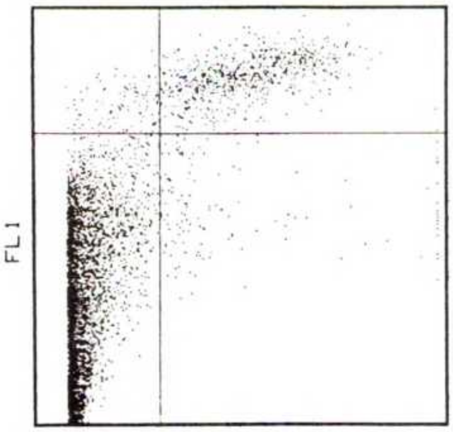

Fig 2. Distribution of megakaryocyte-enriched marrow cells by fluorescence (FL1) *v* FSC. The horizontal line represents the fluorescence gate setting to select the highly fluorescent cells. The vertical line represents the FSC gate setting to distinguish between small (<20 μm) and large megakaryocytes.

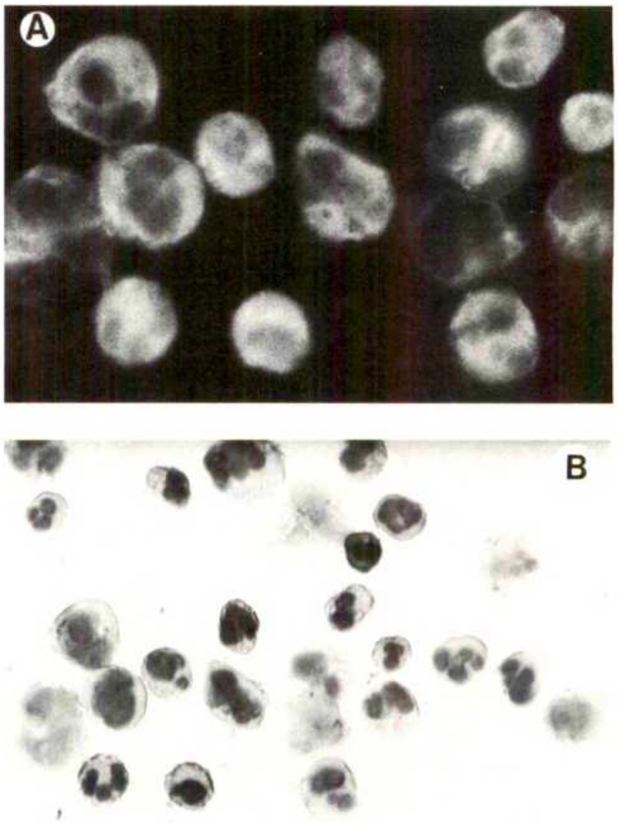

Fig 3. Highly fluorescent large cells sorted according to the gate settings shown in Fig 2. Cells were sorted directly onto glass slides, fixed, and viewed under a fluorescence microscope (A) (original magnification × 400; current magnification × 280). The same preparation was stained with hematoxylin (B) (original magnification × 250; current magnification × 176).

Table 2. Ploidy Distribution of Small and Large Megakaryocytes

Megakaryocytes	Ploidy					
	2N	4N	8N	16N	32N	64N
Small	29.3*	27.4	20.2	19.0	4.1	
Large	4.2	7.9	12.1	40.6	30.0	5.2

Small and large cells were defined as described in the text. Four thousand small cells and 14,000 large cells were examined for this study.

*Percentage of total.

estimated to be 15.2 ± 4.8 μm (n = 4,000). Because the relationship between FSC and the size of large cells had not been established, the mean diameter of unfixed large cells was measured microscopically and averaged 34.2 ± 11.8 μm (n = 150). Table 2 demonstrates the ploidy distribution of both the large and the small megakaryocytes as measured by propidium iodide staining. The modal ploidy of the large cells was 16 (40.6%), with 30.0% of cells being 32N, whereas about 60% of the small cells were 2N to 4N.

Cell culture. Megakaryocytes were cultured in liquid medium in microwell plates or slide chambers (Fig 4, culture of large cells). The viability, as assessed by trypan blue dye exclusion, ranged between 50% for the large cells and 75% for the small ones. Cells were maintained in culture more than three days. Twenty-four hours after the initiation of culture, only 50% to 70% of the cells appeared to be viable, showing a round shape, refractile cytoplasm, and nondiscernable nuclear outline.

To examine the biosynthetic ability of the isolated cells, incorporation of ^{3}HTdR and a ^{3}H-leucine-lysine mixture into TCA-precipitable material was measured. Thymidine incorporation was significantly higher by the small cells than by the large ones ($P < .05$; Table 3). The incorporation rate of all megakaryocytes was between that of the small and the large cells. The addition of hypoxanthine and aminopterin to the culture medium resulted in a six- to sevenfold increase in the incorporation rate. A low incorporation rate was observed in plasma-free medium, although the effect of aminopterin supplementation to this medium was not tested (data not shown). No increase in cell number during the culture period was observed. The relative incorporation rate of amino acids by the different cell types was similar to that observed with thymidine (Table 4) and was significantly lower in plasma-free medium (data not shown). As shown in Table 5 for untreated cells at time 0 and 20 hours, the addition of either radiolabeled precursor 20 hours after the initiation of culture resulted in a reduction of the incorporation rate by 30% to 50%.

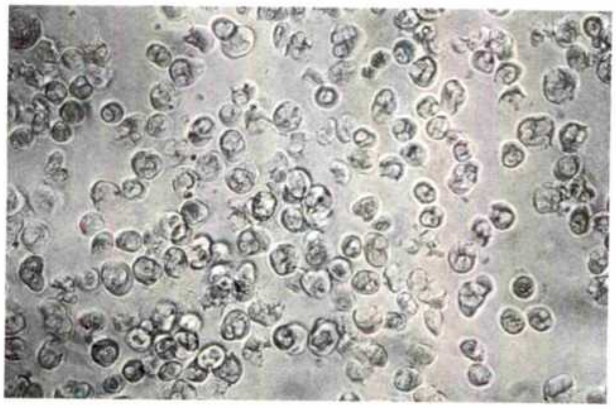

Fig 4. Phase-contrast photomicrograph of sorted large megakaryocytes after 24 hours in culture (original magnification × 200; current magnification × 150).

Table 3. ^{3}HTdR Incorporation by Isolated Megakaryocytes

Culture Conditions	Cells		cpm (± 1 SD)	cpm/Cell (Mean)
	Type	Number		
10% plasma	Small	1,500	1,950 ± 282	1.3
	Large	2,000	1,560 ± 211	0.8
	All	3,000	3,357 ± 349	1.1
10% plasma + AH*	Small	1,500	13,842 ± 1,633	9.2
	Large	1,500	7,212 ± 1,175	4.8
	All	650	4,225 ± 548	6.5

Cells were cultured in 200 μL of the specified medium and labeled with 2 μCi ^{3}HTdR for 20 hours. Cells were harvested into 10% cold TCA, filtered through GF/C glass fiber filters, washed, and the precipitable material was counted. Background counts of samples containing no cells were subtracted. The average background was 185 cpm.

*Aminopterin (A) was added to a final concentration of 4×10^{-7} mol/L and hypoxanthine (H) to 1×10^{-4} mol/L.

Response to GM-CSF. To determine whether isolated megakaryocytes would respond to hematopoietic growth factors, the response of megakaryocytes to rGM-CSF in culture was examined. Only the small megakaryocytes responded with significant increments in the thymidine or amino acid incorporation rates (2.3- and 1.5-fold respectively, $P < .01$; Table 5) with no siginificant change in the incorporation rate by the large cells (data not shown). The effect was pronounced only in cultures that were treated with the factor for about 20 hours before pulse-labeling with the radioisotopes. No increase in cell number was observed during the culture period.

DISCUSSION

Purified viable human megakaryocytes are required to study some aspects of the biology of these cells such as biosynthesis of specific proteins or the response to growth factors. Based on recent studies demonstrating the utility of fluorescence-activated cell sorting for analysis of animal megakaryocytes,[5,6,9-11] we have established a technique for

Table 4. Amino Acid Incorporation by Isolated Megakaryocytes

Culture Conditions	Cells		cpm (± 1 SD)	cpm/Cell (Mean)
	Type	Number		
10% plasma*	Small	2,000	15,040 ± 1,095	7.5
	Large	2,000	10,520 ± 1,237	5.2
	All	2,000	12,020 ± 2,209	6.0

Cells were cultured in 200 μL medium and pulse-labeled with 2 μCi mixed ^{3}H-leucine and ^{3}H-lysine (1:1) for 20 hours. The cells were then harvested, and TCA-precipitable material was counted. Background counts of samples containing no cells were subtracted. The average background was 470 cpm.

*Lysine, leucine-deficient RPMI 1640 medium supplemented with 10% dialyzed normal human plasma.

Table 5. Incorporation of ^{3}HTdR and ^{3}H-Lysine-Leucine Into Megakaryocyte Cultures Treated With rGM-CSF

GM-CSF Treatment	Time of Addition of Radiolabel	Cells Plated	Radiolabeled Precursor	cpm (± 1 SD)
–	0	2,000	^{3}HTdR	18,000 ± 2,969
+				19,510 ± 2,725
–		1,500	^{3}H-Leu/Lys	10,510 ± 1,334
+				9,820 ± 1,519
–	20	2,000	^{3}HTdR	15,420 ± 3,049
+				36,080 ± 6,500
–		1,500	^{3}H-Leu/Lys	5,075 ± 681
+				7,830 ± 1,028

Small megakaryocytes were cultured in 200 μL of either Iscove's medium plus AH for ^{3}HTdR incorporation or leucine- lysine-deficient RPMI 1640 medium for ^{3}H-amino acid incorporation, both supplemented with 10% normal human plasma. Recombinant GM-CSF was added at 1 U/mL at the outset of culture. The radiolabeled precursors were added immediately or 20 hours after the addition of rGM-CSF. Eighteen hours later the cells were harvested, and TCA-precipitable material was counted as described. The data represent means (± 1 SD) of three experiments.

the preparative sorting of human megakaryocytes. Using this method, we have shown that megakaryocytes (*a*) can be isolated on the basis of an intrinsic cell surface marker rather than physical characteristics, (*b*) can be characterized and separated further by size, (*c*) are biosynthetically intact with the small cells more active than the large cells, and (*d*) respond to at least one characterized hematopoietic growth factor, GM-CSF.

The method described herein presents several advantages. (*a*) Bone marrow aspirates are used as the starting material, thereby avoiding dependence on amputated bones and permitting serial studies to investigate cell physiology as well as clinical disorders of megakaryocytopoiesis. (*b*) The technique ensures a nearly pure and biosynthetically viable (50% to 70%) cell population. (*c*) Selection of megakaryocytes by size, ploidy, or the expression of specific surface markers can be made for analytic as well as growth regulation studies.

To sort megakaryocytes, monocellular suspensions are critical. The major obstacle in the preparation of a single-cell suspension of human marrow megakaryocytes was cellular aggregation. Any clot formation during marrow aspiration exacerbated this phenomenon. To obviate cell clumping, a number of modifications of the collection and dilution media were made. An ACD-A, EDTA, and PGE_1 mixture was used for marrow collection. The citrated dilution medium was supplemented with BSA and PGE_1. However, the adenosine was omitted, and the concentration of theophylline was reduced to improve viability. The marrow suspension was admixed with Percoll to 1.020 g/mL before the density centrifugation, and the collected cell fraction was washed in the presence of a higher concentration of BSA (6%). These changes resulted in optimal dispersion and preservation of cells. Nevertheless, in some experiments detectable cellular aggregation was observed due to some additional, as yet undefined mechanism(s).

The recovery of megakaryocytes was dependent on the number of washing steps and the total time required for the purification procedure. The presorting steps required two to 2.5 hours and resulted in a 50% yield of the enriched marrow cells. The efficiency of sorting was dependent on the accuracy of the optical and fluidic settings. At optimal settings an efficiency of 80% to 90% was obtained. Surprisingly, concentrating the purified megakaryocytes in the collecting tube by centrifugation resulted in about a 50% loss. To increase the yield and to improve viability, megakaryocytes were subsequently sorted directly into culture vessels. As a result, the overall yield from an average bone marrow aspirate (about 4×10^8 cells) under optimal sorting conditions was increased to 80,000, which constituted about 40% of the initial number. Higher yields of human megakaryocytes have been reported. However, the use of marrow aspirates for separation by CCE resulted in approximately 63% recovery but only 19% purity.[18] Amputated ribs offer a higher starting number of cells[3] with less admixture with blood. Separation by density followed by two velocity centrifugations resulted in up to 85% recovery and 70% to 95% purity.[1,16] The need for ribs and the selection of cells on the basis of size limits to some degree the applicability of that method.[2,3,12] Although higher recoveries have been reported by others, the major aim of this work was to optimize the sorting technique to obtain pure and viable cells for subsequent studies in culture. It is likely that with optimization of the presorting steps the final yield would be significantly improved. However, as shown by the incorporation studies, the present yield is sufficient to permit studies of growth factor responsiveness.

The purity of the isolated megakaryocytes approached 100% as observed in Figs 3 and 4. Achievement of this purity is governed by the specificity of the cell labeling and the accuracy of the sorting settings. As previously shown, the monoclonal antibody we used has been shown to react only with platelets and megakaryocytes in marrow.[21] Although epitopes of GPIIb and IIIa are found in other cell types, those studies indicated that the monoclonal antibodies used in the present study identify epitopes found in platelets and megakaryocytes but not in endothelial cells or monocytes.[21] The ability to distinguish between megakaryocytes and other marrow cells is demonstrated in Figs 1 and 2 where the mean fluorescence level of the megakaryocytes was more than 50-fold that of the control cells. Using bidimensional plots of fluorescence *v* FSC, the frequency of megakaryocytes (about 2%) enables localization and selection of the population of interest by more accurate gate settings. The precision of sorting can then be evaluated in real time by sorting the large, highly fluorescent cells on slides and examining them by phase-contrast microscopy to ascertain that these cells are morphologically recognizable megakaryocytes.

Sorting of small megakaryocytes requires higher resolution because it is based on fluorescence only and there is no sharp demarcation between small low-fluorescent megakaryocytes and other cells exhibiting low nonspecific fluorescence such as autofluorescence (Fig 1A). Use of the F(ab′) fragment improves the resolution by augmenting the difference in fluorescence intensity between megakaryocytes and control cells up to 180-fold (Fig 1B). By using bidimensional plots, small megakaryocytes were sorted according to the FL1 level of the total megakaryocytic population and to the FSC levels corresponding to the size up to 20 μm in diameter

(Fig 2, upper left quadrant). To verify the lineage association of these small, highly fluorescent cells, we examined the expression of GPIb by an immunoenzymatic staining technique. Approximately 75% of the small GPIIb/IIIa-positive cells also stained for GPIb. Based on data that GPIIb/IIIa appears or is detectable earlier in the differentiation sequence than GPIb, and on our previous data assessing the specificity of our anti-GPIIb/IIIa monoclonal antibodies,[21,25] we conclude that these small cells expressing high levels of GPIIb/IIIa represent early cells in the megakaryocytic lineage that have yet to express the GPIb epitope.

As shown by us[21] and others,[11] the majority (70%) of small megakaryocytes are found at densities >1.050 g/mL. The choice of cells of density <1.050 g/mL in this study was dictated by the need for megakaryocyte-enriched marrow to establish the sorting method. Studies requiring greater numbers of small cells could be performed by increasing the Percoll density in the preliminary enrichment step. It is possible, however, to eliminate the enrichment procedure if sorting time is not a limitation.

The size range of megakaryocytes (10 to 46 μm) is similar to that reported by others,[3,18,21] and the ploidy distribution is similar to that found in previous work[21] except for the higher proportion of 2N and 4N cells, probably as a result of using a different technique.

Human megakaryocytes have the capacity to grow in culture and to be biosynthetically active.[1,18] However, cultures of highly purified human megakaryocytes have not been examined. In the present selection method the isolated pure megakaryocytes were able to grow in medium supplemented with 10% plasma for at least three days and to show biosynthesis of proteins and DNA. An attempt to replace plasma by a defined medium was not fully successful, thereby indicating the inadequacy of the plasma substitute. Further studies will be required to determine whether this problem is trivial or related to the absence of a required growth factor(s). The low incorporation rate by the large cells suggests that mature megakaryocytes synthesize macromolecules at a lower rate than the small cells. However, it cannot be excluded that large cells were damaged during the isolation procedure to a greater extent than the small cells.[11] The incorporation rate was decreased by 30% to 50% when cultures were pulse-labeled after 20 hours, which reflects, at least in part, the decrease in cell viability as a function of time in culture. A similar phenomenon was observed by Greenberg et al using CCE and density centrifugation to purify rat megakaryocytes.[12]

As has been found for other marrow cells, megakaryocytic proliferation and maturation are controlled by hematopoietic growth factors.[19] Pure cultures of marrow-derived megakaryocytes provide the opportunity to study the direct effect of such factors on the maturation of these cells. In this study the effect of rGM-CSF was examined. The data showed that the small cells responded with a significant increase in thymidine and amino acid incorporation. The insignificant response of the large cells may reflect biologic nonresponsiveness of mature megakaryocytes or could be related to damage incurred during the isolation procedure. The response of the small cells became evident when the cells were preincubated for 20 hours with rGM-CSF. This delay may be due to a requirement for synthesis of a regulatory protein(s) before the onset of general protein synthesis or recovery of cellular responsiveness to GM-CSF because they were exposed to nonphysiological medium for several hours. It is conceivable that GM-CSF is stimulating the megakaryocyte colony–forming cell (CFU-MK) or possibly a myeloid progenitor cell is contaminating the cultures. Some investigators have suggested that CFU-MK express GPIIb/IIIa, which permits these cells to be sorted by our method.[26] Mitigating against these possibilities are (*a*) the lack of an increment in cell number during the culture period, (*b*) the infrequency of progenitor cells (at least in mice) at Percoll densities <1.050 g/mL,[13] and (*c*) more recent evidence suggesting that CFU-MK do not express GPIIb/IIIa.[27] Nonetheless, GM-CSF appears to promote the biosynthesis of macromolecules in nonproliferating, presumably endoreduplicating megakaryocytes. This conclusion is not entirely surprising because GM-CSF has been shown to promote proliferation of progenitor cells of other lineages including the CFU-MK as well as to influence the functions of mature cells in the granulocytic lineage.[28-30]

Based on these results and the known analytic potential of fluorescence-activated cell sorting, we conclude that the present method provides a useful means for the isolation and analysis of human megakaryocytes. This technique may facilitate the study of normal megakaryocytopoiesis in humans as well as the investigation of various clinical disorders of thrombocytopoiesis.

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Augmentation of Neutrophil-Mediated Erythrocyte Lysis by Cells Derived In Vitro From Human Monocytes

By Franco Dallegri, Alberto Ballestrero, Guido Frumento, and Franco Patrone

Neutrophilic polymorphonuclear leukocytes (PMNs) were incubated with opsonized zymosan and lysed human erythrocytes (RBCs) as measured by a ^{51}Cr release method. Conversely, myeloperoxidase (MPO)-negative hydrogen peroxide (H_2O_2)-generating cells, derived in vitro from human monocytes (monocyte-derived cells (MDCs), were ineffective per se but capable of augmenting the lysis by PMNs. The lysis by PMNs and PMNs plus MDCs was inhibited by catalase, azide, taurine, and alanine, consistent with the requirement for hypochlorous acid (HOCl). As detected under conditions similar to those used for lytic assays, MDCs failed to produce HOCl but augmented the HOCl recovery from the PMN-RBC system. Moreover, when the extent of the lysis was plotted as a function of the HOCl recovery, a positive linear relationship was found. Although the actual size of the H_2O_2 extracellular pool could not be measured because of the inexistence of a reliable assay to probe our cytolytic model without perturbing the equilibrium of the system, the results presented suggest that MDCs enhance the PMN-mediated lysis by improving the HOCl production, presumably by supplying extra amounts of H_2O_2 to be handled by PMN MPO. In fact, the events mediated by MDCs could be reproduced by using an appropriate H_2O_2-generating enzymatic system (glucose–glucose oxidase). The present study provides direct evidence for the possibility of cooperation between MPO-positive and MPO-negative phagocytes in exerting functions (HOCl production and, in turn, cytolysis) possibly relevant to the outcome of inflammatory processes.

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WHEN TRIGGERED by appropriate stimuli, either soluble (phorbol myristate acetate) or particulate (opsonized zymosan [OPZ], heat-aggregated IgG), neutrophils (neutrophilic polymorphonuclear leukocytes [PMNs]) have been shown to be capable of lysing a wide spectrum of eukaryotic target cells.[1-5] These in vitro systems of cytolysis have been used to understand the mechanisms by which PMNs may injure tissue cells at inflammatory sites. In the present paper, by the use of a previously characterized system constituted of OPZ-triggered PMNs and human erythrocytes (RBCs) as targets,[6] we provide evidence that the PMN cytolytic efficiency can be augmented by myeloperoxidase (MPO)-negative cells derived in vitro from human monocytes (MDCs) and per se incapable of mediating lysis. The results uncover a novel possible role for MPO-negative mononuclear phagocytes in interaction with PMNs during inflammatory processes.

MATERIALS AND METHODS

Preparation of PMNs. Heparinized venous blood (heparin, 10 U/mL, Liquemin, Roche, Milan, Italy) was obtained from healthy male volunteers. PMNs were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient as previously described.[6] Contaminating erythrocytes were removed by hypotonic lysis.[6] The resulting PMNs were washed three times with Hanks' balanced salt solution lacking phenol red (HBSS, Flow Laboratories, Ltd, Irvine, Scotland) and resuspended in HBSS supplemented with 0.1 mg/mL bovine serum albumin (BSA, Calbiochem-Behring, San Diego) (BSA-HBSS). Final cell suspensions contained 97% or more PMNs and more than 98% viable cells as evaluated by the ethidium bromide–fluorescein diacetate test.[7]

Preparation of MDCs. Venous blood (350 mL) from healthy male volunteers was collected into 53 mL of anticoagulant citrate-phosphate-dextrose-adenine (CDPA) (Baxter, Trieste, Italy). The buffy coat, obtained two to three hours after venipuncture, was diluted with an equal volume of 0.9% NaCl, and 25 mL of the cell suspension was layered on 15 mL Ficoll-Hypaque (Nyegaard & Co, Oslo) in 50-mL polypropylene conical tubes (No. 25330, Corning Glass Works, Corning, NY). After centrifugation (400 g, 30 minutes, 22°C), the upper cell layer (mononuclear cells) was collected and spun down at 400 g for ten minutes at 22°C. The sedimented cells were washed three times with HBSS (200 g, ten minutes, 22°C) and resuspended in Fisher's medium (Flow) supplemented with 25% filtered horse serum (HS, Flow), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (HS-Fisher's medium[8]). The cells were then plated in 75-cm^2 tissue culture flasks (Sterilin, Ltd, Teddington, England) in thin suspension layers (2×10^7/mL, 5 mL). After incubation for two hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2, nonadherent cells were removed by washing five times with prewarmed (37°C) HBSS and the adherent cells overlaid with 8 mL HS-Fisher's medium. The flasks were then returned to the CO_2 incubator. Three days later, the medium containing detached cells was decanted, and adherent cells were rinsed three times with prewarmed (37°C) HBSS. Adherent cells were then removed by pipetting after incubation (20 minutes, 4°C) with phosphate-buffered saline (PBS, pH 7.4) containing 0.2% EDTA (Sigma Chemical Co, St. Louis) and 5% heat-inactivated (30 minutes, 56°C) fetal calf serum (Flow).[9] The resulting cells were centrifuged (200 g, ten minutes, 4°C), washed three times with cold (4°C) HBSS, and resuspended in cold (4°C) BSA-HBSS. Only cell suspensions containing >90% α-naphthyl acetate esterase–positive cells, as determined by the method of Li et al,[10] were used. These cells, referred to as MDCs, were >97% viable as judged by the ethidium bromide–fluorescein diacetate test.[7]

Preparation of OPZ. OPZ was prepared by incubating a suspension containing 10 mg/mL boiled zymosan (Sigma) in PBS (pH 7.4) with an equal volume of fresh human serum for 30 minutes at 37°C. The particles were then washed three times with PBS and resuspended in BSA-HBSS at the appropriate concentration.

Measurement of RBC lysis. The RBC lysis was studied by a

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^{51}Cr release method as previously described.[6] Briefly, RBCs were washed four times with HBSS and labeled (60 minutes, 37°C) with 100 to 200 μCi $Na_2[^{51}Cr]O_4$ (Radiochemical Centre, Amersham, England). The cells were then washed three times with HBSS and resuspended in BSA-HBSS. When required, RBCs were pretreated (60 minutes, 37°C) with 20 mmol/L 3-amino-1,2,4-triazole (AT, Sigma) plus 2 mmol/L L-ascorbic acid (Sigma) (RBC^{AT}) to inhibit catalase activity.[11] A quantity of 10^5 RBCs were mixed with PMNs, MDCs, or PMNs plus MDCs at appropriate concentrations. In some experiments, MDCs were replaced by glucose oxidase (GO, type II, Lot 111F-9605, Sigma) dissolved in HBSS (prepared without glucose) and mixed with the other components of the reaction to reach a final concentration of 5 mU/mL (incubation medium, BSA-HBSS with 1 mg/mL glucose). Incubations were initiated by the addition of OPZ (0.4 mg/mL) and followed by centrifugation of the samples at 50 g (five minutes, 22°C). Tests were performed in triplicate and in a final volume of 200 μL in round-bottomed microplates (Sterilin). After incubation for four hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2, the ^{51}Cr release was determined in the cell-free supernatants. The percentage of cytolysis (percent ^{51}Cr release) was calculated according to the formula $(E - S)/(T - S) \times 100$, where E is the counts per minute released in the presence of effector cells, T is the counts per minute released after lysing target cells with 5% Triton X-100 (Sigma), S is the counts per minute spontaneously released by target cells in the absence of effector cells (in each case, <10%).

Measurement of H_2O_2 release. The hydrogen peroxide (H_2O_2) release was studied by a modification of the homovanillic acid (HVA)–horseradish peroxidase (HRP) method[12] as previously described.[11] Tests were performed in triplicate under experimental conditions similar to those used for cytolytic assays except that incubations were done in Falcon plastic tubes (12 × 75 mm, Falcon Labware, Oxnard, CA). Briefly, 5×10^5 PMNs were incubated (37°C) with and without 0.4 mg/mL OPZ in the presence of 200 μmol/L HVA (Sigma) plus 2 U/mL HRP (Sigma). When required, 2×10^6 RBC targets were added. Similar experiments were carried out with an appropriate number of MDCs both in presence and in absence of PMNs. The cells were sedimented by centrifugation (50 g, five minutes) at the beginning of the assay. The reactions, performed in a final volume of 2 mL, were stopped after 20 minutes with 0.25 mL of 0.1 mol/L glycine-NaOH buffer (pH 12) containing 25 mmol/L EDTA. The amount of H_2O_2 in the cell-free supernatants was determined fluorimetrically ($\lambda_{ex} = 312$ nm, $\lambda_{em} = 420$ nm) by using appropriate standards of H_2O_2. Preliminary experiments showed that the amount of H_2O_2 recovered from OPZ-triggered neutrophils incubated (20 minutes) as a pellet equals that recovered from OPZ-triggered neutrophils incubated as a pellet during the first five minutes of the assay period (20 minutes) and then resuspended and maintained in suspension.

Measurement of HOCl production. The generation of hypochlorous acid (HOCl) by OPZ-triggered PMNs was quantitated by the taurine trapping technique[13] as previously described.[11] The conditions of the assay were identical to those used for the H_2O_2 release except that the reactions were carried out in a final volume of 1 mL. Briefly, an appropriate number of PMNs was incubated (20 minutes, 37°C) with 0.4 mg/mL OPZ in the presence of 20 mmol/L taurine (Sigma). Tests were performed in the presence and in the absence of 2×10^6 RBC targets or 300 units catalase (bovine liver, Sigma; amount of enzyme chosen on the basis of preliminary experiments). Similar experiments were carried out with MDCs both in presence and in absence of PMNs. The cells were sedimented by centrifugation (50 g, five minutes) at the beginning of the assay. At the end of the incubation period (20 minutes), catalase (600 units, Sigma) was added to reduce any residual H_2O_2. The samples were then incubated (22°C, five minutes) with 100 nmol of 5-thio-2-nitrobenzoic acid (Nbs) prepared by reducing 5-5′-dithiobis (2-nitrobenzoic acid) (Sigma) as described by Aune and Thomas.[14] The amounts of Nbs oxidized were determined by measuring the absorbance at 412 nm of the cell-free supernatants ($\epsilon = 1.36 \times 10^4$ mol/L/cm). The quantities of HOCl trapped by taurine (yelding taurine chloramine) were calculated by taking into account that 1 mol taurine chloramine oxidizes 2 mol Nbs.[13] As for the H_2O_2 assay, the method of HOCl detection maintained its efficiency also when tests were carried out with cells incubated as a pellet for 20 minutes. Under the conditions described earlier, the uptake of taurine chloramine by RBCs, via the 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS)-inhibitable anion transport system,[15] did not affect the taurine chloramine recovery (the amounts of taurine chloramine recovered in the presence of RBCs equalled those recovered in the presence of RBCs pulsed (30 minutes) with 0.1 mmol/L DIDS (Sigma).[11]

Enzyme assays. The total cell content of MPO was measured after lysing the cells by sonication followed by freezing-thawing and solubilization with 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma).[16] The MPO activity released by cells incubated (one hour, 37°C) with 0.4 mg/mL OPZ was determined in HTAB-untreated cell-free supernatants. The MPO assay was done by an *O*-dianisidine method as previously described.[11] One unit of enzyme activity was defined as that oxidizing 1 μmol of *O*-dianisidine/min at 25°C (optical density, 550, $\epsilon = 11.3$ mmol/L/cm).

For determination of RBC catalase activity, the cells were lysed as described by Beutler,[17] and the enzyme activity was determined by recording the rate of decomposition of H_2O_2 at 230 nm ($\epsilon = 0.071$ mmol/L/cm).[17] The activity of "reagent" catalase (bovine liver, Sigma) was measured similarly. The results were expressed as nanomoles H_2O_2/min at 25°C or units (1 unit of enzyme activity was defined as that catalyzing the breakdown of 1 μmol H_2O_2/min at 25°C).

Special materials. Superoxide dismutase (SOD, type 1, bovine blood, Sigma), stored at −20°C at a concentration of 5 mg/mL in distilled water, was diluted in medium before use. Catalase (bovine liver, 40,000 U/mg protein), taurine, L-alanine, mannitol, and benzoate were purchased from Sigma. Sodium azide (NaN_3) and $(NH_4)_2SO_4$ were from Merck, Darmstadt, FRG. MPO, as a supernatant from the PMN granule fraction (MPO 4,800 mU/mL, 24.9 mg/mL protein), was prepared by using normal human PMNs and stored at −20°C.[18] Other reagent-grade compounds were used as obtained from commercial suppliers. In some experiments, BSA-HBSS medium was replaced with a Cl^--free, 0.1 mol/L phosphate buffer (pH 7.4) with 1.5 mmol/L $MgSO_4$ supplemented with 1 mg/mL glucose (Merck) and 0.1 mg/mL BSA (Calbiochem-Behring)(Cl^--free medium).

RESULTS

As depicted in Fig 1, OPZ-triggered PMNs lysed RBC targets, whereas MDCs were ineffective. When added to the PMN-RBC system (0.25×10^5 PMNs plus 10^5 RBCs, PMN:RBC ratio, 1:4), MDCs enhanced the lysis in a dose-dependent manner (Fig 2). As detailed in Table 1, the lysis in the complete PMN-MDC-RBC system (0.25×10^5 PMNs plus 0.25×10^5 MDCs plus 10^5 RBCs) was inhibited by adding catalase to destroy H_2O_2 (heat-inactivated catalase was ineffective), azide to inhibit MPO, and amino acids (alanine, taurine) to scavenge HOCl. Scavengers of superoxide anion (SOD) and hydroxyl radicals (mannitol, benzoate) were ineffective (Table 1). Similar results were obtained in the PMN-RBC system (Table 1), in agreement with our previous findings.[6,11] Moreover, the absence of Cl^- completely prevented lysis in the PMN-MDC-RBC system (percent ^{51}Cr release, 0.1 ± 0.8 and 36.0 ± 2.3 in the absence and

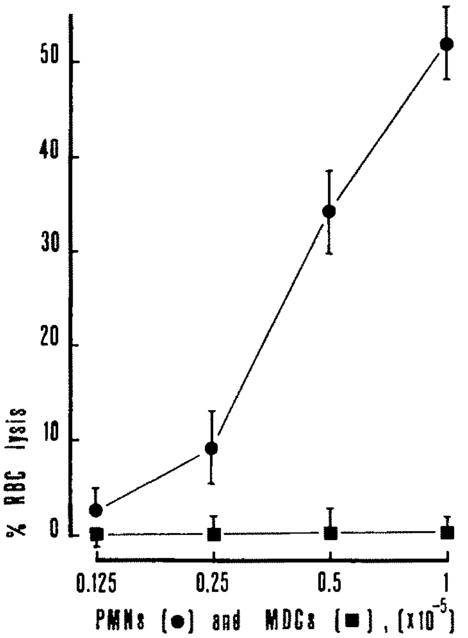

Fig 1. OPZ-triggered RBC lysis by PMNs or MDCs. The number of RBCs is 10^6/well. Each point represents x̄ ± 1 SD of at least three experiments.

in the presence of Cl^- respectively, x̄ ± 1 SD, n = 3). Similarly, Cl^- was required for RBC lysis by PMNs.[19] Finally, the addition of 20 mmol/L NH_4^+ caused >95% inhibition of the lysis in both the PMN-RBC and the PMN-MDC-RBC systems. These data suggest that amplification of PMN-mediated lysis by MDCs requires HOCl production via the MPO-H_2O_2-Cl^- system.

To gain further insight into the mechanism whereby MDCs enhance PMN-mediated lysis, we measured the recovery of H_2O_2 and HOCl generated by PMNs and MDCs under conditions similar to those used for cytolytic assays (cells incubated as a pellet). As shown in Table 2, both PMNs and MDCs, incubated with OPZ in absence of RBCs, released H_2O_2. Nevertheless, only PMNs produced HOCl (Table 2), consistent with the absence of detectable MPO activity in MDCs (MDC MPO content, undetectable; PMN MPO content, 157.2 ± 11.3 mU/10^6 PMNs, OPZ-triggered MPO release, 4.2 ± 0.6 mU/10^6 PMNs/1h, x̄ ± 1 SD, n = 4). When MDCs were added to PMNs in absence of RBCs, the H_2O_2 recovery increased, but the HOCl recovery did not change (Table 2). It seems therefore that the HOCl production cannot be further enhanced by the extra amount of H_2O_2 supplied by MDCs. This is in agreement with the incapacity of the H_2O_2-generating enzymatic system, glucose-GO, to increase the HOCl production by OPZ-triggered PMNs (HOCl recovery, 15.5 ± 2.6 and 15.9 ± 2.6 nmol/5 × 10^5 PMNs/20 min from PMNs incubated alone or with 5 mU/mL GO respectively; 5 mU/mL generated GO 2.3 ± 0.5 nmol/H_2O_2/min, x̄ ± 1 SD, n = 3). In ancillary experiments carried out with cells in suspension instead of centrifuged as a pellet, the HOCl production by OPZ-triggered PMNs could not be augmented by adding 5 mU/mL GO or 16 mU/mL MPO from the supernatant of the PMN granule fraction (92.8 ± 6.9, 92.8 ± 7.3, and 91.5 ± 9.2 nmol HOCl/10^6 PMNs/1 h from PMNs incubated alone, with GO, and with MPO respectively, x̄ ± 1 SD, n = 3).

When RBCs were added to PMNs to complete the system (PMN:RBC ratio, 1:4; number of RBCs 2 × 10^6; cells were incubated as a pellet) as in the cytolytic assays, a decreased

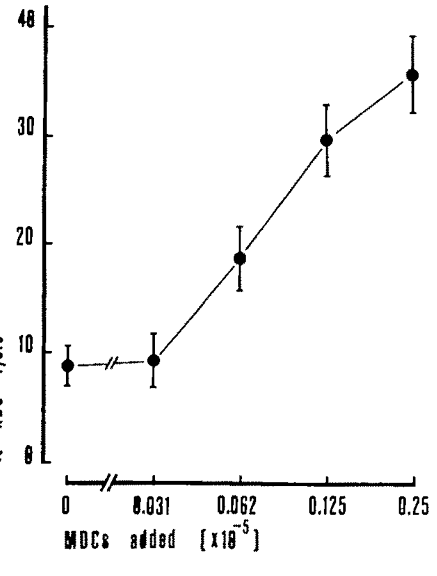

Fig 2. Augmentation of the OPZ-triggered PMN-mediated RBC lysis by MDCs. The abscissa shows the number of MDCs added to the PMN-RBC system (0.25 × 10^6 PMNs plus 10^6 RBCs/well). Each point represents x̄ ± 1 SD of at least four experiments.

Table 1. Effect of Scavengers and Inhibitors of Oxygen-Reactive Species on the OPZ-Triggered RBC Lysis by PMNs Plus MDCs or by PMNs

Additive	Percent RBC Lysis* by	
	PMNs + MDCs	PMNs
None	35.6 ± 2.5	49.3 ± 5.0
SOD, 300 U/mL	35.6 ± 4.0	47.3 ± 2.3
Catalase, 4,000 U/mL	0.1 ± 1.1	−0.1 ± 0.7
Azide, 1 mmol/L	5.6 ± 2.7	8.0 ± 4.2
Taurine, 20 mmol/L	4.6 ± 1.8	5.9 ± 2.5
Alanine, 20 mmol/L	4.5 ± 2.5	4.5 ± 3.1
Mannitol, 20 mmol/L	35.1 ± 4.2	47.0 ± 2.6
Benzoate, 20 mmol/L	34.8 ± 3.7	50.9 ± 7.4

*PMN-MDC-RBC system, 0.25 × 10^6 PMNs plus 0.25 × 10^6 MDCs plus 10^6 RBCs; PMN-RBC system, 10^6 PMNs plus 10^6 RBCs. Results are expressed as x̄ + 1 SD of three experiments.

Table 2. Recovery of H_2O_2 and HOCl From OPZ-Triggered PMNs and/or MDCs in the Presence and in the Absence of RBCs

Assay Conditions*	Extracellular Recovery†	
	H_2O_2	HOCl
PMNs	15.3 ± 2.7	14.8 ± 1.2
MDCs	7.4 ± 1.0	0
PMNs + MDCs	21.3 ± 3.5	14.1 ± 1.4
PMNs + RBCs	7.6 ± 1.3	8.1 ± 0.9
MDCs + RBCs	4.6 ± 1.7	0
PMNs + MDCs + RBCs	12.3 ± 1.4	12.8 ± 1.2

*PMNs, 5 × 10^5; MDCs, 5 × 10^5; RBCs, 2 × 10^6.
†In nmol/20 min, x̄ ± 1 SD of three experiments.

H_2O_2 and HOCl recovery (~50%) was found (Table 2). This reduction in HOCl generation results in a relative impairment of the HOCl-dependent PMN lytic efficiency.[6] As summarized in Table 2, MDCs increased the H_2O_2 and HOCl recovery from the PMN-RBC system. Moreover, when the extent of the lysis by PMNs and PMNs plus MDCs (Figs 1 and 2) was plotted as a function of the HOCl recovery detected under conditions similar to those used for cytolysis (identical cell ratios but with 2 × 10^6 RBCs instead of 10^5 RBCs used in lytic assays), a positive linear relationship was found (Fig 3). Together, these results suggest the possibility that MDCs amplify the lysis primarily by improving the HOCl production in the PMN-RBC system, possibly through an effective restoration of the PMN H_2O_2 extracellular pool depleted by RBC targets. Three additional findings are consistent with this possibility. First, the replacement of RBC targets with RBC^{AT} (RBC^{AT} had a >94% reduction in catalase activity; catalase activity of native RBCs 4,258 ± 112 nmol H_2O_2/min/10^6cells, $\bar{x} \pm 1$ SD, n = 6), incapable of efficiently consuming PMN-derived H_2O_2,[6] completely abrogated the MDC capacity to augment the PMN-mediated HOCl-dependent (not shown) lysis (percent increment, 1.1 ± 3.7, $\bar{x} \pm 1$ SD, n = 3) and the HOCl recovery from the system (percent increment, −0.02 ± 8.2, $\bar{x} \pm 1$ SD, n = 4). Second, enhancement of the HOCl production by MDCs in the PMN-RBC system (Table 2) could be reproduced by substituting catalase for RBCs in the system (Table 3) and choosing an amount of enzyme (300 units in 1 mL) that results in a reduction of the HOCl recovery from PMNs similar in magnitude to that observed in the presence of 2 × 10^6 RBCs (Tables 2 and 3). The requirement for 300 units catalase, ie, ~35 times the amount of catalase in 2 × 10^6 RBCs, is conceivably due to the fact that the enzyme is distributed throughout the assay volume (1 mL) whereas RBC catalase is confined to the cell pellet (the HOCl assays were carried out with cells as a pellet to simulate the conditions used for measuring the lysis). Third, the PMN-mediated RBC lysis was augmented by 5 mU/mL GO, per se virtually ineffective (Fig 4). The lysis was prevented by the MPO inhibitor azide as well as by the HOCl-trapping agent taurine (Fig 4). In addition, 5 mU/mL GO enhanced the recovery of HOCl from the PMN-RBC system by 46.9% + 5.2% ($\bar{x} \pm 1$ SD, n = 3), against an expected value of 55% (value calculated on the basis of the regression line from Fig 3 by using the extent of the RBC lysis by PMNs plus GO as known variables).

Table 3. Recovery of HOCl From OPZ-Triggered PMNs and/or MDCs in the Presence and in the Absence of Catalase

Assay Conditions*	HOCl Recovery†
PMNs	15.6 ± 1.9
MDCs	0
PMNs + MDCs	15.7 ± 1.3
PMNs + catalase	8.8 ± 1.4
MDCs + catalase	0
PMNs + MDCs + catalase	14.3 ± 1.0

*PMNs, 5 × 10^5; MDCs, 5 × 10^5; catalase, (bovine liver, Sigma), 300 units.

†In nmol/20 min, $\bar{x} \pm 1$ SD of three experiments.

DISCUSSION

In our model system as reported elsewhere[6] and herein confirmed by probing cytolysis with chemical agents, the RBC lysis by PMNs requires HOCl production via the MPO-catalyzed oxidation of chloride ions by H_2O_2. Although the cytolysin or cytolysins actually operative remain to be identified,[11,20,21] the extent of the lysis strictly correlates with the amount of HOCl generated by PMNs, at least under our experimental conditions (the present paper and Dallegri et al[6]). Consistent with this view, MPO-negative MDCs incapable of generating HOCl did not cause lysis unless supplemented with MPO activity from a super-

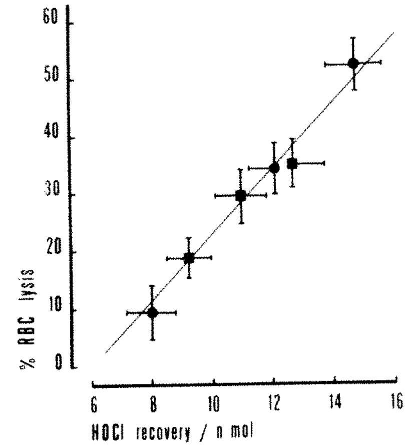

Fig 3. Positive linear relationship ($P < .01$) between the RBC lysis mediated by PMNs (●) and PMNs plus MDCs (■) (data from Figs 1 and 2) and the HOCl recovery (nmol/20 min) from PMNs (●) and PMNs plus MDCs (■) in presence of RBC targets (as for the lytic assays, the number of RBCs was kept constant, 2 × 10^6). Each point represents $\bar{x} \pm 1$ SD of at least three experiments. Regression line, y = 5.436 × −31.266.

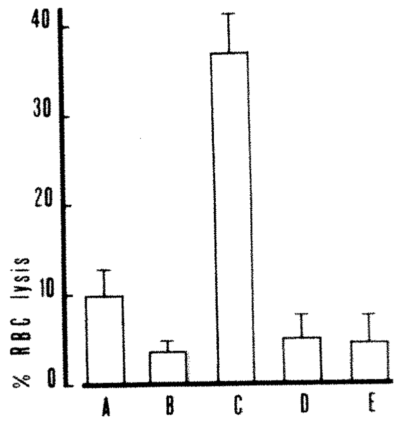

Fig 4. Effect of GO on the RBC lysis by OPZ-triggered PMNs. The ordinate shows the percent lysis by (A) 0.25 × 10^6 PMNs, (B) 5 mU/mL GO, (C) 0.25 × 10^6 PMNs plus 5 mU/mL GO, (D) 0.25 × 10^6 PMNs plus 5 mU/mL GO plus 1 mmol/L azide, and (E) 0.25 × 10^6 PMNs plus 5 mU/mL GO plus 20 mmol/L taurine. The number of RBCs is 10^6/well. Results are expressed as $\bar{x} \pm 1$ SD of three experiments.

natant of the PMN granule fraction (unpublished observations). Nevertheless, they efficiently augmented both HOCl recovery from the PMN-RBC lytic system and the HOCl-dependent lysis.

A series of interconnected events underlies the expression of the observed MDC "helper" activity. After the interaction between the PMN surface and OPZ, a plasmalemma-associated NADPH oxidase system is activated in the area of PMN-OPZ contact,[22] with consequent H_2O_2 production. The generated H_2O_2 is released directly into the extracellular fluid until the OPZ particle is completely engulfed. Part of this H_2O_2 can probably undergo backdiffusion into PMNs themselves.[23] At the luminal surface of the phagosomal membrane, which is derived from the plasmalemma, H_2O_2 production continues,[24] and the generated H_2O_2 can diffuse into PMN cytoplasm and outside the cell.[25] The net amount of H_2O_2 released extracellularly (extracellular H_2O_2 pool) represents that fraction of H_2O_2 synthesized and not used by PMN catabolic pathways in different subcellular compartments, including MPO within phagosomes[5,21] and the catalase and glutathione cycle within the cytosol.[26-28] Although discrepancies exist among different authors,[13,29] in our setting the extracellular H_2O_2 pool is likely to be transformed entirely into HOCl by the MPO released into the medium or absorbed to the PMN surface. In fact, HOCl production by OPZ-triggered PMNs could not be augmented by adding an appropriate surplus of MPO activity, which suggests that the H_2O_2 available for the chlorinating reaction is entirely used by the released MPO. Moreover, HOCl production could not be augmented by biochemical (GO) or cellular (MDCs) extra sources of H_2O_2, which suggests that the amount of MPO released functions at a maximum rate without a potential reserve of activity. Thus, it appears that OPZ-triggered PMNs produce a definite amount of HOCl by releasing MPO and H_2O_2 via a process so balanced that excesses of MPO and H_2O_2 are virtually absent or, if present, trivial at least under our conditions (pH, Cl^- concentration, temperature). Although the H_2O_2 released and transformed into HOCl represents a relatively small fraction of the total amount of H_2O_2 produced (Nauseef et al,[26] Test and Weiss,[27] and unpublished observations) and is generally assumed to be in dynamic equilibrium with the remainder (primarily dissipated by the PMN catabolic pathways mentioned earlier), it can be expected that the addition of extra H_2O_2-consuming pathways to the PMN microenvironment can perturb the equilibrium of the system so much that subsequent HOCl production is impaired. RBCs added to OPZ-triggered PMNs to make up a complete cytolytic system reduce the HOCl production by consuming H_2O_2 primarily via catalase (Dallegri et al,[6] Test and Weiss,[27] and the present paper). Such a consumption of H_2O_2, which results in a relative impairment of HOCl-dependent PMN lytic efficiency,[6] is likely to take place within RBC targets before lysis. In fact, the PMN-delivered lethal hit and the RBC catalase–mediated impairment of the lysis occur during the first 45 minutes of incubation when target cells are still "viable" (changes in the final extent of the lysis could not be detected when exogenous catalase was added to block the lytic pathway 45 minutes after the start of the incubation period, lysis at this time being virtually absent; unpublished observations). This is consistent with results obtained by other authors in a different system[30] and with the fact that H_2O_2, having a relatively low reactivity and no charge, can diffuse away from its sites of formation and across cell membranes easily.[31] Although attempts to directly measure the actual size of the extracellular H_2O_2 pool in our cytolytic system with cells as a pellet (without adding extra H_2O_2-consuming pathways, for instance, HRP) are presently hampered by the nonexistence of a reliable H_2O_2 assay, the results herein reported provide inferential evidence that the reduction of the PMN extracellular H_2O_2 pool by RBC catalase and the reconstitution of this pool by MDCs are crucial to MDC-mediated augmentation of PMN HOCl production and, in turn, lysis. This is suggested by different findings. First, neither HOCl recovery nor the extent of the lysis could be augmented by MDCs after inhibition of the RBC H_2O_2–consuming capacity, ie, using RBC^{AT} as targets. Second, enhancement of HOCl production by MDCs in the PMN-RBC system could be reproduced by substituting an appropriate amount of reagent catalase for RBCs in the system. Third, MDC-dependent enhancement of both HOCl recovery from the PMN-RBC system and PMN-mediated lysis could be reproduced by replacing MDCs with an appropriate H_2O_2-generating enzymatic system (GO, Fig 4) or H_2O_2-generating, granule-depleted PMN cytoplasts obtained as described by Roos et al[32] and unpublished observations). Fourth, the "final" events occurring in our system in the presence and in the absence of MDCs (amount of HOCl produced and extent of the lysis) strictly correlate (Fig 3). Thus, on the basis of these observations coupled with the evidence that MDCs are per se cytolytically ineffective and with the results obtained by probing the lytic systems with chemical agents, we conclude that (*a*) MDCs amplify the lysis by improving HOCl production, presumably via an effective restoration of the extracellular H_2O_2 pool generated by PMNs and depleted by RBCs; and (*b*) if present, the intervention of nonoxidative cytolytic factors, released by MDCs and capable of contributing to the lysis,[33] would be trivial at least under the present conditions. In addition, the involvement of PMN-directed cytokines released by MDCs and capable of increasing the HOCl production can be ruled out because MDCs did not affect the HOCl recovery from PMNs in absence of RBCs. Similarly, the possibility that MDCs may act by releasing NH_4^+, which favors NH_2Cl formation ($NH_4^+ + HOCl \rightarrow NH_2Cl + H_2O + H^+$),[34] can be ruled out because exogenous NH_4^+ inhibited the lysis in the PMN-RBC system thus suggesting that lytic levels of NH_2Cl cannot be reached in our conditions. Finally, because MDCs had no effect when added to the PMN-RBC^{AT} system, it is unlikely that they act by sensitizing RBC targets to the PMN-delivered, HOCl-mediated attack.

In conclusion, it has been reported that macrophages ingesting effete PMNs or granules from PMNs become capable of mediating MPO-dependent iodination.[35] Further, macrophages can use eosinophil peroxidase (EPO) to enhance their microbicidal or cytolytic activity.[36-38] These results have suggested a way by which MPO-negative and MPO-positive phagocytes can interact synergistically in exerting certain effector functions. Nevertheless, the aforementioned experiments were performed with cellular debris or purified cellular components (EPO, MPO). In the present study using two complete viable cellular systems, ie, PMNs and MPO-negative MDCs, we attained direct evidence for cooperation between two distinct populations of phagocytes in mediating cytolysis. Although speculative, the present use of MDCs as a model raises the possibility that macrophages at inflammatory sites may interact with neutrophils to increase the local HOCl production and, in turn, the injury of surrounding tissue cells. This might be only one of the events amplified by macrophages and relevant to the outcome of the inflammatory process. In fact, aside from the role in promoting cytolysis, phagocyte-derived HOCl and/or its chlorinated derivatives have recently been recognized to mediate a variety of biologic effects, either protective or harmful to the host,[21,39] ranging from the destruction of microorganisms[40] and modulation of lymphocyte function[41] to the inhibition of antiproteinase activities[42] and inactivation of chemotactic factors.[43]

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Expression of Myelomonocytic Antigens on Chronic Lymphocytic Leukemia B Cells Correlates With Their Ability to Produce Interleukin 1

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We analyzed the expression of myelomonocytic-associated antigens on lymphocytes from B cell chronic lymphocytic leukemia (B-CLL) patients. Blood mononuclear cells were depleted of monocytes by one-step Percoll density gradient centrifugation and tested for antigen expression by fluorescent microscopy and flow cytometry. The reactivity of patient lymphocytes was as follows: 26 of 31 were positive for CD14 (Myr), 22 of 31 for a monocyte Fc receptor (MFC-1), 22 of 31 for CD11b (C3bi receptor), eight of 31 for CD15 (Leu-M1), five of 18 for CD13 (My 7), seven of 18 for My 9, and five of 30 for Mo 2. The B lymphocytes of B-CLL patients were also tested for the ability to produce interleukin 1 (IL-1) after depletion of monocytes and T lymphocytes. In 13 of 17 cases, B lymphocytes of patients produced IL-1 as detected in a mouse thymocyte proliferation assay and, in selected cases, a radioimmunoassay specific for IL-1_β. The 13 cases that produced IL-1 were also positive for the expression of one or more myelomonocytic-associated antigens, whereas the four cases that did not produce IL-1 lacked expression of these antigens. In conclusion, the malignant B cells of B-CLL patients frequently express a variety of antigens generally considered specific for myelomonocytic cells, and expression of these antigens is associated with the ability to produce IL-1.

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THE CLONAL ORIGIN of B cell chronic lymphocytic leukemia (B-CLL) is well established,[1-4] and various degrees of B cell maturation have been consistently detected among clones from different patients as well as among cells of each individual clone.[5-8] Differences in the expression of some surface antigens and receptors exist between B-CLL cells and resting, mature peripheral blood B cells. Notably, B-CLL cells express the 67-kd glycoprotein CD5 recognized by the monoclonal antibodies Leu-1 and OKT1.[9-11] This marker is found on all T cells but is absent from most B cells from adult blood and tissues, with the exception of a minority of B cells localized in the follicles of lymphoid tissues.[12] In contrast, CD5 is frequently detected on fetal B cells and in the early phase of B cell reconstitution after bone marrow transplantation.[13,14] Altogether, these data suggest that the normal counterpart of B-CLL cells is an immature B cell. Other surface determinants unrelated to normal B cell lineage have been found on B-CLL cells such as antigens revealed by anti–T cell heteroantisera[15]; Leu-8–TQ1, an antigen previously thought to be restricted to cells of T lineage[16]; and sheep erythrocyte receptors, commonly, but not always related to antisheep erythrocyte activity of the monoclonal surface immunoglobulin.[17-20]

The finding of myeloid lineage markers on malignant lymphoblasts has been quite frequent in acute leukemia, which raises the issue of lineage infidelity or promiscuity.[21-28] In contrast, myeloid marker expression on malignant cells from chronic leukemia patients has not been described, with the exception of C3bi (CR3) receptors (CD11b), which have been detected on B-CLL cells[29] and, more commonly, on hairy cells.[30] In this respect, it is of note that CD11b is also found on cells with the T-helper[31] and T-suppressor[32] phenotypes and on a large proportion of natural killer (NK) cells.[33] B-CLL cells may share functional properties with myelomonocytic cells and NK cells, namely, the ability to produce and release interleukin 1 (IL-1).[34-37]

In this study we show that, in addition to CD11b, other myelomonocytic markers are frequently expressed on B-CLL cells and that the presence or absence of these markers correlates with their ability or inability to produce IL-1.

MATERIALS AND METHODS

Patient population. Thirty-one patients with B-CLL were diagnosed on the basis of clinical, morphological, and immunologic criteria. All patients had WBC counts ranging from 11 to 200 × $10^3/\mu L$ (85% to 95% lymphocytes). Hematologic data and surface marker analyses of the malignant cells are shown in Table 1. Eleven patients had not received treatment for their disease; the remainder had been treated discontinuously with chlorambucil and prednisone but were free of therapy for a month or longer before study. Blood samples for these studies were obtained after informed consent.

Phenotypic analysis of B-CLL cells. Peripheral blood mononuclear cells were separated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and depleted of monocytes by one-step density gradient centrifugation on 50.6% Percoll (Pharmacia) in RPMI 1640.[38] This monocyte-depleted cell fraction was used for phenotypic analyses with monoclonal antibodies obtained from Becton Dickinson (Mountain View, CA) (Leu-1, Leu-5, Leu-M1, HLA-DR), Coulter Immunology (Hialeah, FL) (B1, B4, Mo1, Mo2, My4, My7, My9) and Ortho Pharmaceutical Corp (Raritan, NJ) (OKM1). The MFC-1 monoclonal antibody was produced in our laboratory. The specificities of these antibodies are shown in Table 2. Monoclonal antibody binding was assessed by indirect immunofluorescence using an affinity-purified, fluorescein isothio-

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Table 1. Clinical and Immunologic Data of Patients With Active B-CLL

Patient No.	Rai Stage	Previous Treatment	Immunoglobulin Expression*	Percentage of Positive Cells†				
				Leu-1	Leu-5	B1	B4	HLA-DR
1	III	Yes	$\gamma\kappa$	9	20	67	68	71
2	I	Yes	κ	87	12	55	43	73
3	I	Yes	$\mu\delta\kappa$	36	10	80	92	93
4	0	No	ND	78	25	59	67	66
5	0	No	ND	93	38	52	67	75
6	I	Yes	ND	49	24	33	76	84
7	I	No	—	33	9	74	76	79
8	I	Yes	κ	75	38	63	69	80
9	0	No	—	97	6	82	83	91
10	II	Yes	—	5	5	91	ND	89
11	I	Yes	$\mu\delta\kappa$	91	12	93	87	88
12	0	No	λ	89	30	90	78	81
13	III	Yes	$\mu\delta\kappa$	84	12	76	85	83
14	IV	Yes	$\mu\delta\kappa$	93	30	54	67	89
15	IV	Yes	—	73	13	80	93	91
16	II	No	$\mu\delta\kappa$	98	5	84	95	97
17	IV	Yes	κ	90	12	67	88	88
18	II	No	λ	94	11	88	90	78
19	I	No	κ	96	3	80	92	90
20	III	Yes	$\mu\kappa$	86	12	84	91	85
21	IV	Yes	$\mu\delta\kappa$	65	24	69	73	76
22	IV	Yes	$\mu\delta\kappa$	62	11	87	78	89
23	I	No	$\mu\delta\kappa$	65	29	54	59	63
24	0	No	$\mu\delta\kappa$	58	39	68	59	87
25	III	Yes	$\gamma\kappa$	95	12	ND	ND	85
26	I	No	$\mu\kappa$	98	3	96	93	94
27	0	No	$\mu\delta\kappa$	82	33	51	31	59
28	II	Yes	$\mu\kappa$	82	49	57	59	64
29	0	No	$\mu\delta\kappa$	93	8	96	85	96
30	I	No	$\mu\delta\kappa$	87	65	82	83	87
31	I	No	$\gamma\lambda$	87	70	93	95	98

Abbreviation: ND, not determined.

*In some cases cells stained too weak to permit identification of immunoglobulin.

†As determined by fluorescence-activated cell sorting (FACS) analysis.

cyanate (FITC)-labeled goat antimouse immunoglobulin antiserum (Southern Biotechnology Associates, Birmingham, AL) as a secondary reagent. Nonspecific staining was assessed with irrelevant, FITC-conjugated mouse IgG1 and IgG2a antibodies (Becton Dickinson) and with the FITC-labeled secondary reagent alone. Before staining with monoclonal antibodies, the cells were incubated with aggregated Ig to block binding to Fc receptors. This procedure was followed with all monoclonal antibodies except MCF-1, which is specific for the 66 to 68-kd Fc receptor of IgG.[48] Two-color immunofluorescence was performed by using phycoerythyrin (PE)-conjugated My4 (Coulter) and FITC-conjugated Leu-16 (Becton Dickinson). Cell samples were analyzed by flow cytometry (FACS-Analyzer, Becton Dickinson) and by fluorescence microscopy.

IL-1 production and assay. The blood mononuclear cells of

Table 2. Monoclonal Antibodies

Antibody	Antigen Cluster Designation	Reactivity	References
Leu-1	CD5	Thymocytes, mature T lymphocytes, some B cells	11,12,39,40
Leu-5	CD2	Thymocytes, mature T lymphocytes (sheep erythrocyte receptor associated)	41
HLA-DR		Monocytes/macrophages, activated T lymphocytes, B cells, myeloid and erythroid precursors, dendritic cells	42
B1/Leu-16	CD20	B lymphocytes	43
B4	CD19	B lymphocytes and their precursors	44
Leu-M1/MMA	CD15	Myeloid cells, monocytes and macrophages, activated T lymphocytes	45
My4	CD14	Monocytes	46,47
My7	CD13	Myelomonocytic cells and their precursors	46,47
My9		Myelomonocytic cells and their precursors	46,47
MFC-1		Monocyte IgG Fc receptor	48
Mo2		Monocytes	49
OKM1/Leu-15/Mo1	CD11	Monocytes, granulopoietic cells, some T cells, NK cells (C3bi receptor)	31,32,49-51

patients were first depleted of monocytes by one-step Percoll gradients[38] and then depleted of T cells by rosetting with 2-aminoethylisothiouronium–treated sheep erythrocytes.[52] The resulting B-CLL cell suspensions consistently contained <1% T cells and <2% monocytes as determined by morphological and cytochemical criteria.[53]

As a control for IL-1 production, monocytes were isolated from the mononuclear cells of ten normal subjects by one-step Percoll gradients.[38] The B-CLL cell preparations and monocytes from healthy donors were cultured at 5×10^5 cells/mL in RPMI 1640 medium with 5% fetal calf serum (FCS) in round-bottomed culture tubes. Each cell suspension was cultured with culture medium alone, with 2 μg/mL lipopolysaccharide (LPS, *Escherichia coli*, Sigma Chemical Co., St Louis), and LPS with the addition of 1 μg/mL indomethacin (Sigma). After a 24-hour incubation at 37°C in a humidified atmosphere with 5% CO_2, culture supernatants were harvested after centrifugation and stored at −70°C until assay for IL-1 activity or prostaglandin E_2 (PGE_2) levels.

The IL-1 activity of supernatants was determined by a mouse thymocyte proliferation assay as described.[54] Thymic cell suspensions were obtained from 4- to 8-week-old C3H/HeJ mice, suspended in RPMI 1640 medium supplemented with 3% FCS and 2×10^{-5} mol/L 2-mercaptoethanol, and plated at 1.5×10^6 thymocytes/well in 96-well flat-bottomed plates. Dilutions of test supernatants were added to triplicate wells with and without the addition of a submitogenic concentration of phytohemagglutinin (PHA, Wellcome Reagents, Durham, NC) to reach a final volume of 200 μL/well. Cultures were incubated for 72 hours at 37°C and pulsed with 0.5 μCi/well of ^{3}H-thymidine (^{3}H-TdR) ten hours before harvesting on a multiple automated sample harvester. The ^{3}H-TdR uptake was determined by β-scintillation counting. IL-1 activity is reported as counts per minute of thymocyte cultures containing test supernatants and PHA minus the counts per minute of thymocyte cultures with test supernatants alone. Some supernatants were also analyzed for levels of IL-1_β by radioimmunoassay (RIA) (Cistron Biotechnology, Pine Brook, NJ).

Supernatants were analyzed for levels of PGE_2 by RIA as described previously.[55]

Table 3. Myelomonocytic Markers on B-CLL Lymphocytes

Patient No.	OKM1*	MCF-1	Mo2	My4	My7	My9	Leu-M1
1	+	+	−	+	+	+	+
2	−	+	−	+	−	−	−
3	+	+	−	+	−	−	−
4	+	−	−	+	−	−	−
5	−	−	−	−	−	−	−
6	−	−	−	+	−	−	−
7	−	−	−	−	−	−	−
8	+	−	+	+	+	+	+
9	+	+	+	+	−	+	+
10	−	+	−	+	ND	ND	−
11	+	+	ND	+	ND	ND	+
12	+	+	+	+	ND	ND	+
13	+	+	−	+	ND	ND	−
14	+	+	−	+	ND	ND	−
15	−	−	−	−	ND	ND	−
16	+	+	−	+	ND	ND	−
17	−	+	−	+	ND	ND	−
18	+	+	−	+	ND	ND	+
19	+	+	−	+	ND	ND	−
20	+	+	−	+	ND	ND	−
21	+	−	−	+	−	+	−
22	+	+	−	+	+	−	−
23	−	+	−	−	−	−	−
24	−	−	−	−	−	−	−
25	+	+	−	+	−	−	−
26	+	+	−	+	−	−	−
27	+	+	−	+	ND	ND	−
28	+	+	−	+	ND	ND	−
29	+	−	−	+	−	+	−
30	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+

*Surface marker analyses were determined on monocyte-depleted peripheral blood lymphocytes by both FACS and immunofluorescence microscopy. Positivity (+) is indicated when >40% of cells were stained by the specific marker.

RESULTS

Thirty-one patients with B-CLL were studied and classified according to the Rai clinical staging system (Table 1). Although 52% of the patients had received prior treatment, we detected a high percentage of circulating monoclonal B lymphocytes in these cases, as shown by the expression of CD19, CD20, and HLA-DR determinants and the restriction to a single light-chain surface immunoglobulin. In some cases, immunoglobulin was undetectable on the cell surface. The B-CLL cells of 29 to 31 patients expressed CD5, the p67 antigen that is detected on a subpopulation of normal and neoplastic B lymphocytes.[9-11]

The expression of myelomonocytic-associated antigens on B-CLL lymphocytes was analyzed with a panel of monoclonal antibodies, the specificity of which is listed in Table 2. Lymphocytes from most of the patients expressed the CD14 antigen (My4), whereas the expression of CD13 (My7) and My9 deteminants was not as common (Table 3). The CD15 antigen recognized by Leu-M1 was also expressed on approximately 20% of patient cell samples. Finally, in 22 of 31 patients, the lymphocytes expressed C3bi or Fc receptors, not necessarily coincidentally, as shown by their reactivity with OKM1 or MFC-1 monoclonal antibodies. The FACS profiles of B-CLL cells stained for a B cell and a monocytic antigen from three representative patients are shown in Fig 1. The top and middle rows illustrate the equivalent CD19 and CD14 reactivity on lymphocyte preparations from two patients. The bottom row shows the FACS profiles of lymphocytes from a patient whose malignant cells lacked CD14. Several cases were studied by two-color immunofluorescence to directly demonstrate coexpression of the myelomonocytic marker (CD14) and the B cell marker (CD20) on the same population of cells. A representative contour plot is shown in Fig 2 where 80% of the lymphocytes were positve for both CD14 and CD20. The specificity of binding was assessed by incubating lymphocytes with irrelevant FITC-conjugated mouse IgG1 and IgG2a monoclonal antibodies and with FITC-conjugated goat antimouse immunoglobulin alone. In all cases, lymphocytes were negative for immunofluorescence after these treatments.

B lymphocytes from 17 B-CLL patients were tested for IL-1 production. Mononuclear cells from these patients were depleted of monocytes and T lymphocytes and cultured for 24 hours in the absence or presence of LPS and LPS with indomethacin (Table 4). Monocyte-enriched populations

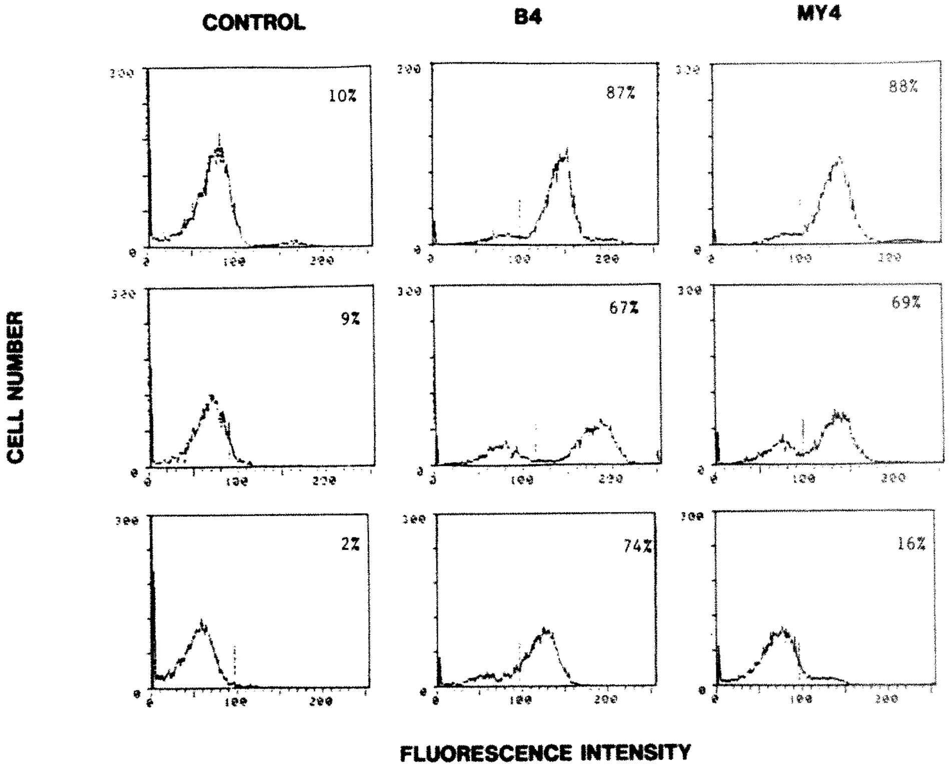

Fig 1. FACS-Analyzer profiles demonstrating expression of a B cell antigen (B4, CD19) and a monocytic antigen (My4, CD14) on B-CLL cells from three representative patients. In cases illustrated in the top and middle rows the distribution of cells coexpressing the two antigens is virtually identical. Cells from the patient illustrated in the bottom row express CD19 but not CD14.

from ten normal subjects were used as positive controls for IL-1 production. B lymphocytes from most of the patients studied spontaneously produced low levels of IL-1 as detected in a mouse thymocyte proliferation assay. LPS was able to enhance IL-1 production in all but four cases. We also assessed IL-1 activity in supernatants generated in the presence of both LPS and indomethacin, a prostaglandin synthetase inhibitor. Monocytes produce both IL-1 and PGE_2, and PGE_2 in culture supernatants can inhibit mouse thymocyte proliferation in the assay for IL-1 activity,[56] thus masking the actual levels of IL-1 produced by the cells as measured in this assay. Although the addition of indomethacin trebled the mean counts per minute of IL-1 activity in monocyte-LPS culture supernatants, it had little or no effect on IL-1 activity detectable in supernatants from most B-CLL lymphocyte cultures. Supernatants were also tested for PGE_2 levels by RIA (data not shown). Although monocyte culture supernatants generated with LPS had detectable levels of PGE_2 (usually 2 to 10 ng/mL), the addition of indomethacin reduced the amount of PGE_2 produced to <0.01 ng/mL. In contrast, LPS-stimulated B-CLL cell supernatants had <0.01 ng/mL PGE_2, with or without the addition of indomethacin.

Because the mouse thymocyte proliferation assay is not entirely specific for IL-1 but also can detect other mitogenic factors, we also tested for the presence of IL-1_β in a specific RIA. Culture supernatants from CD14+ B lymphocytes of six B-CLL patients were positive for IL-1 in both the mouse thymocyte proliferation assay and the IL-1_β-specific RIA.

We examined the relationship between myelomonocytic antigen expression and IL-1 production by LPS-stimulated B lymphocytes from B-CLL patients. Of the 17 cases studied for both parameters, four cases had little or no IL-1 production (<5,000 cpm) under any culture condition tested. These cases were also negative for all of the myelomonocytic antigens tested (CD14, CD13, My9) as well as for CD15, C3bi, and Fc receptor expression (Tables 3 and 4, patients no. 5, 7, 15, and 24). On the contrary, the 13 patients whose B lymphocytes produced moderate or high amounts of IL-1 activity as compared with monocytes were CD14 (My4)-positive and also randomly displayed other myelomonocytic markers. These associations are summarized in Table 5.

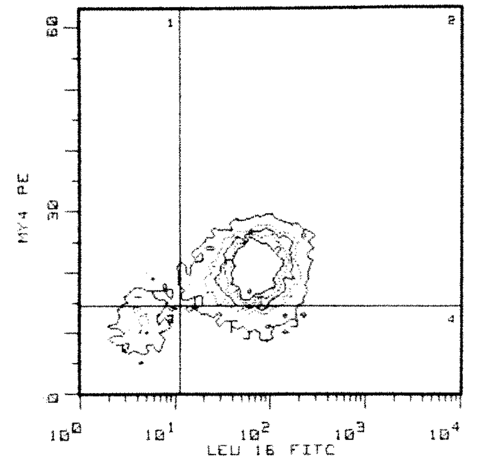

Fig 2. A FACS contour plot demonstrating coexpression of the B cell antigen CD20 (Leu-16) and the monocyte antigen CD14 (My4) on the same population. In this case 80% of the cells were positive for both antigens by two-color immunofluorescence, whereas 9% were positive for FITC-Leu-16 only and 3% were positive for PE-My4 only.

Table 4. IL-1 Activity in Culture Supernatants of B-CLL B Lymphocytes

Patient No.	Mouse Thymocyte ^{3}HTdR Incorporation (cpm)*		
	Medium Only†	LPS (2 μg/mL)	LPS + Indomethacin (1 μg/mL)
1	5,029	20,136	26,333
2	7,407	8,939	24,808
3	9,731	17,580	31,814
5	3,750	3,002	3,893
6	7,943	12,976	24,844
7	3,138	3,994	4,141
8	4,670	7,674	11,993
10	5,754	41,417	48,751
12	434	8,002	15,560
13	3,398	19,221	15,519
15	1,339	3,863	3,515
16	100	6,280	9,701
17	822	9,949	9,609
24	110	1,503	2,814
25	1,796	8,976	11,609
26	211	8,682	13,655
27	5,595	12,624	15,761
Normal monocytes (Mean ± SE)	13,053 ± 3,357	16,337 ± 2,674	45,226 ± 8,221

*Counts per minute of ^{3}HTdR in thymocyte cultures containing 25% test supernatant and PHA minus counts per minute of culture containing test supernatant alone.

†Test supernatants were obtained from B-CLL cells or monocytes from healthy donors cultured in medium alone, with LPS, or with LPS and indomethacin as described in Materials and Methods.

DISCUSSION

In this study we have shown that, in a high percentage of cases, B-CLL cells bear surface C3bi receptors (CD11b) that are not found commonly on blood or tissue B cells. In addition they have a 66- to 68-kd Fc receptor for IgG similar to that of monocytes and express a variety of membrane antigens considered specific for myelomonocytic cells (most frequently My4/CD14) and not detected previously on malignant B cells.[46,47]

The presence of C3bi receptors on B-CLL cells is not completely unexpected because this determinant has been observed on hairy cells.[30] It is possible that, in addition to activated (hairy) B cells, CD11b is expressed on a small subset of blood or tissue B cells, possibly also CD5+. Sharing of similar Fc IgG receptors among cells of different lineages is also not unprecedented, as shown by the detection of CD16 on NK cells and granulocytes.[57] Also, some B cell lines, eg, SB, have been shown to have a low-density expression of the 66- to 68-kd monocyte Fc receptor on their surface.[58]

The variate but frequent expression of myelomonocytic lineage antigens on B-CLL cells provides another criterion to determine the phenotypic heterogeneity of the neoplastic cell clones in a disease characterized by a high degree of clinical uniformity. A correlation between expression of myelomonocytic markers and clinical stage of disease, white cell count, or previous treatment was not apparent. In contrast, an association was noted between the presence of these antigens and the ability of the cells to produce IL-1. Thus, aberrant antigens were, in these cases, good indicators of a functional characteristic of the neoplastic cells. B-CLL cells that were shown to be poor IL-1 producers (four of 17 cases) lacked all the myelomonocytic antigens tested as well as CD11b and the monocyte-associated 66- to 68-kd Fc receptor. The inability to produce IL-1 was unrelated to treatment because, in three of four cases, the patients had not received previous treatment and the monocytes were inefficient IL-1 producers in all four cases.

IL-1 production by normal peripheral blood B lymphocytes and B-CLL cells has been described previously.[34-37] Here we have shown that IL-1 production by B-CLL cells is increased after LPS stimulation and, in a few cases, further boosted by incubation with LPS and indomethacin. In three cases the addition of indomethacin produced a two- to threefold increase of IL-1 activity in the culture supernatants compared with that observed in the supernatants of cells incubated with LPS alone. This effect of indomethancin is probably not attributable to prostaglandin production by contaminating monocytes because the amount of PGE$_2$ pro-

Table 5. Relationship Between IL-1 Production and Myelomonocytic Markers on B Lymphocytes From 17 B-CLL Patients

IL-1 Activity*	Number of Cell Samples Reactive With Monoclonal Antibodies†							
	My4	My7	My9	Leu-M1	Mo2	Mo1	OKM1	MFC-1
19,996 ± 11,178 (9,609-48,751)	13/13	2/7	2/7	5/13	2/13	5/13	5/13	11/13
3,580 ± 578 (2,814-4,141)	0/4	0/3	0/3	0/4	0/4	0/4	0/4	0/4

*Mean counts per minute ± SD (range) of ^{3}HTdR uptake in thymocyte cultures containing 25% test supernatant (from LPS and indomethacin cultures) and PHA minus the counts per minute of cultures with test supernatant alone.

†Not all patients were studied with respect to My7 and My9 expression.

duced in cultures of both LPS and LPS+ indomethacin-stimulated B-CLL cells was very low. We have previously demonstrated that indomethacin can exert an immunomodulatory effect that is not related to the inhibition of prostaglandin synthesis.[55] Therefore it is possible that indomethacin exerted a direct effect on IL-1 production by B-CLL cells.

Neoplastic cells from lymphoid malignancies commonly express immunoglobulin and/or other surface antigens that allow lineage assignment and determination of the stage of maturation or activation.[4] However, mixed-lineage cell phenotypes (eg, lymphoid and myeloid) have been described in hematopoietic malignancies, particularly in acute lymphoblastic leukemia and in lymphoblastic crisis of acute myelogenous leukemia.[21-28] In several instances, this lineage infidelity is simply attributable to a lack of lineage specificity of the antigen tested, as is the case of CD15 (Leu-M1), a hapten that is expressed on myelomonocytic cells and on a variety of neoplastic cells of both hematopoietic and nonhematopoietic origin.[59-66] In other cases, a bona fide mixed phenotype can be determined, and this is reinforced by the finding of inappropriate gene rearrangements (eg, of Ig genes in malignant T lymphoblasts or of Ti_β genes in B cell malignancies).[67-74]

Several hypotheses have been proposed to explain these findings, and they are not mutually exclusive[28]: (*a*) the mixed-lineage phenotype may be a feature of early precursor cells, the extremely low frequency of which prevents identification in the normal; (*b*) the aberrant antigens may be transiently expressed during cell maturation and therefore escape recognition in the normally developing cell population; (*c*) a limited degree of cell promiscuity may precede lineage fidelity as a normal course of the differentiation events; and (*d*) the infidelity of gene expression may be a consequence of the neoplastic transformation. All these possibilities are related to early events during cell differentiation, and the malignant counterparts of early hematopoietic cells commonly produce an acute disease. This may explain the relatively rare finding of aberrant phenotypes in chronic leukemia compared with acute leukemia.

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Long-Term Monoclonal Reconstitution of Erythropoiesis in Genetically Anemic W/W^v Mice by Injection of 5-Fluorouracil–Treated Bone Marrow Cells of $Pgk\text{-}1^b/Pgk\text{-}1^a$ Mice

By Toru Nakano, Noriko Waki, Hidekazu Asai, and Yukihiko Kitamura

The spleen colony-forming assay does not represent the number of hematopoietic stem cells with extensive self-maintaining capacity because five to 50 spleen colony-forming units (CFU-S) are necessary to rescue a genetically anemic (WB X C57BL/6)F$_1$-W/W^v(WBB6F$_1$-W/W^v) mouse. We investigated which is more important for the reconstitution of erythropoiesis, the transplantation of multiple CFU-S or that of a single stem cell with extensive self-maintaining potential. The electrophoretic pattern of hemoglobin was used as a marker of reconstitution and that of phosphoglycerate kinase (PGK), an X chromosome–linked enzyme, as a tool for estimating the number of stem cells. For this purpose, we developed the C57BL/6 congeneic strain with the $Pgk\text{-}1^a$ gene. Bone marrow cells were harvested after injection of 5-fluorouracil from C57BL/6–$Pgk\text{-}1^b/Pgk\text{-}1^a$ female mice in which each stem cell had either A-type PGK or B-type PGK due to the random inactivation of one of two X chromosomes. When a relatively small number of bone marrow cells (ie, 10^3 or 3×10^3) were injected into 200-rad–irradiated WBB6F$_1$-W/W^v mice, the hemoglobin pattern changed from the recipient type (Hbb^d/Hbb^s) to the donor type (Hbb^s/Hbb^s) in seven of 150 mice for at least 8 weeks. Erythrocytes of all these WBB6F$_1$-W/W^v mice showed either A-type PGK alone or B-type PGK alone during the time of reconstitution, which suggests that a single stem cell with extensive self-maintaining potential may sustain the whole erythropoiesis of a mouse for at least 8 weeks.

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THE HEMATOPOIETIC STEM CELL is characterized by extensive self-maintaining capacity and differentiation potential.[1,2] The hematopoietic cell that forms a macroscopic colony in the spleen of either lethally irradiated or genetically anemic W/W^v recipient mice (CFU-S) can differentiate into erythrocytes, granulocytes, megakaryocytes, macrophages,[2,3] and mast cells.[4,5] Therefore, the spleen colony method apparently represents the differentiation potential of the stem cell, but CFU-S are not necessarily considered to have extensive self-maintaining capacity.[6] The latter capacity has been estimated from the proportion of successfully reconstituted animals after transplantation of cell suspensions (presumably containing stem cells) into lethally irradiated or genetically anemic W/W^v recipient mice. For such a reconstitution assay, W/W^v mice are more suitable recipients than lethally irradiated mice because a considerable proportion of mice do not survive lethal irradiation and transplantation of a relatively small number of stem cells. On the other hand, histocompatible stem cells can be transferred to W/W^v mice without preceding lethal irradiation.[7-9]

Wiktor-Jedrzejczak et al[8] and Boggs et al[9] showed that five to 50 CFU-S were necessary to rescue the anemia of a W/W^v mouse. However, both groups of investigators did not determine which was more important, the quantity or the quality of stem cells. In other words, which was the more essential factor, the number of transplanted stem cells or the presence of a particular stem cell with extensive self-maintaining capacity in the injected cell suspension? To answer this question, we examined whether clonal reconstitution occurred after transplantation of a relatively small number of stem cells.

X chromosome–linked alloenzymes have been used as a marker to determine the number of stem cells from which blood cells originate.[10-13] Because of inactivation of one of the two X chromosomes in each somatic cell in females during embryogenesis, only one of two genes for the alloenzymes is active in any single somatic cells. Fialkow examined blood cells of women suffering from chronic myelocytic leukemia (CML) who were heterozygous for the X chromosome–linked gene for glucose-6-phosphate dehydrogenase (G6PD) and hence had two types of enzymatically different somatic cells.[10,11] Although both A- and B-type G6PD were found in normal tissues such as the skin of the CML patients, only a single-enzyme type was found in granulocytes, erythrocytes, and platelets of the patients, which suggests the clonal origin of CML. In mice, X chromosome–linked 3-phosphoglycerate kinase (PGK) alloenzymes can be used to investigate the clonal origin of blood cells.[12,13] In the present study, we developed the C57BL/6 congeneic strain with the $Pgk\text{-}1^a$ gene and used PGK heterozygous C57BL/6–$Pgk\text{-}1^b/Pgk\text{-}1^a$ female mice as donors of hematopoietic stem cells.

Because hematopoietic stem cells surviving a single injection of 5-fluorouracil (5FU) are reported to have more extensive proliferative potential than untreated stem cell populations,[14-16] we used bone marrow cells of 5FU-treated mice as a source of stem cells. When a relatively small number of bone marrow cells (ie, 10^3 or 3×10^3) from 5FU-treated female C57BL/6–$Pgk\text{-}1^b/Pgk\text{-}1^a$ mice were injected into 200-rad–irradiated (WB × C57BL/6)F$_1$-W/W^v (hereafter called WBB6F$_1$-W/W^v) mice, hemoglobin

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pattern changed from the recipient type (*Hbb*^{d}/*Hbb*^{s}) to the donor type (*Hbb*^{s}/*Hbb*^{s}) for at least 8 weeks in seven of 150 mice. Erythrocytes of all these seven WBB6F$_1$-*W*/*W*^{v} mice showed either A-type PGK alone or B-type PGK alone, thereby suggesting that a single stem cell with extensive self-maintaining capacity may sustain the whole erythropoiesis of a mouse at least for 8 weeks.

MATERIALS AND METHODS

Mice. C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} and WBB6F$_1$-*W*/*W*^{v} mice were raised in our laboratory. The original stock of mice with the *Pgk-1*^{a} gene was introduced from Dr Vern M. Chapman, Roswell Park Memorial Institute, to the National Cancer Institute, Tokyo, by Dr Hiroshi Tanooka.[17,18] We obtained C3H mice with the *Pgk-1*^{a} gene from Dr Tanooka and introduced the *Pgk-1*^{a} gene to the C57BL/6 strain of our inbred colony by repeated backcrosses (11 backcrosses at the time of the present study). Because the original C57BL/6 strain has the usual-type PGK gene (*Pgk-1*^{b}), C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice were produced by mating C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{b} female mice with C57BL/6-*Pgk-1*^{a}/Y male mice. Resulting C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice were used as donors of bone marrow cells at 2 to 3 months of age. The A-type/B-type ratio of PGK in erythrocytes of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice is about 50/50.

Although the *W*^{v} mutant gene was derived from the Jackson Laboratory, Bar Harbor, ME, it is now maintained in our inbred C57BL/6 mouse colony.[19] The WB-*W*/+ strain, which was also obtained from the Jackson Laboratory, has been maintained by brother-sister mating. WBB6F$_1$-*W*/*W*^{v} mice that had been produced by a cross between WB-*W*/+ and C57BL/6-*W*^{v}/+ mice, were used as recipients at 4 months of age.

Hemoglobin of WBB6F$_1$-*W*/*W*^{v} mice (*Hbb*^{d}/*Hbb*^{s}) can be distinguished from that of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice (*Hbb*^{s}/*Hbb*^{s}) by electrophoresis.[20,21]

5FU treatment and bone marrow transplantation. 5FU (150 μg/g body weight) was injected intravenously to C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice; the mice were killed four days after the 5FU injection, and bone marrow cells were collected from at least five mice and suspended in Eagle's medium according to the method described previously.[22]

Recipient WBB6F$_1$-*W*/*W*^{v} mice were irradiated (200 rad) with a Shimadzu x-ray machine (180 kV and 20 mA, with a 2-mm Al filter, 50 rad/min) to facilitate the bone marrow reconstitution. Various numbers of bone marrow cells were injected through the lateral tail vein.

Assay of CFU-S. The method of Till and McCulloch[23] was used; the recipient WBB6F$_1$-*W*/*W*^{v} mice were killed eight or 14 days after the transplantation of bone marrow cells; spleens were removed and fixed in Bouin's solution, and colonies were counted with a dissection microscope (7×).

Sampling of blood. Mice were anesthetized with ether, and blood samples were obtained from the retroorbital sinus with heparinized microhematocrit tubes. Care was taken to remove small volumes (<50 μL) of blood to minimize possible hematologic stress. The microhematocrit tube was centrifuged, and the tube was cut beneath the level of the buffy coat; packed erythrocytes were divided into two parts; one part was used for examination of hemoglobin and another part for examination of PGK.

Examination of hemoglobin. Electrophoresis of hemoglobin was done after modification with cystamine according to the method described by Whitney[21]; the hemoglobin pattern was determined visually by using known mixtures as standards. By this method, hemoglobin of WBB6F$_1$-*W*/*W*^{v} mice (*Hbb*^{d}/*Hbb*^{s}) mixed with hemoglobin of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice (*Hbb*^{s}/*Hbb*^{s}) is detectable if the former contributes ≧5% of the total. We assumed that WBB6F$_1$-*W*/*W*^{v} recipient mice were reconstituted by bone marrow cells of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice only when the hemoglobin pattern of the former could not be distinguished from that of the latter. In other words, >95% of erythrocytes in the reconstituted WBB6F$_1$-*W*/*W*^{v} mice were derived from the bone marrow of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice.

Electrophoresis of PGK. Small pieces of filter paper were soaked with blood samples and placed in small plastic tubes; the tubes were kept at −80°C. The PGK pattern of erythrocytes was examined only when WBB6F$_1$-*W*/*W*^{v} recipient mice were assumed to be reconstituted by bone marrow cells of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice. After thawing, pieces of filter paper with blood samples were inserted into the gel plate. The electrophoresis was carried out on a gel plate (10 × 20 cm) of starch (Wako Chemical Co, Tokyo) for 17 hours at 5 V/cm and 4°C according to the method described by Tanooka and Tanaka.[18] The gel plates were then cut into two slices and PGK was detected as nonfluorescent spots due to conversion of NADH to NAD in the PGK assay system. The spot of A-type PGK was usually 6 cm from the origin, and that of B-type was 4 cm from the origin. According to the report of Reddy and Fialkow,[24] the relative activity of PGK alloenzymes was estimated visually by using known mixtures as standards. With this technique, a minor population in a mixture of blood is detectable if it contributes ≧5% of the total PGK activity.

The proportion of A-type PGK in serial blood samples was plotted as the deviation of individual samples from the percentage of A-type PGK in C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice according to Burton et al.[12] Therefore, +50% indicated that only A-type PGK was present, 0% that A-type PGK and B-type PGK were equally mixed, and −50% that only B-type PGK was present.

Limiting-dilution analysis. The principle of the method has been described by Porter and Berry,[25] Breivik,[26] and Boggs et al,[9] and briefly it was carried out as follows. Random samples from a homogeneous cell population were assayed for the presence or absence of stem cells. Such cells should be distributed among samples in a Poisson fashion. The finite probability that a sample will not contain a stem cell is defiend by $P_{(0)} = e^{-\phi x}$ where ϕ is the fraction of stem cells in the sample of x cells. P is the proportion of samples devoid of stem cells and estimates $P_{(0)}$. The concentration of stem cells in the sample can then be expressed as $\phi = -\ln P/x$.

RESULTS

First, we confirmed the effect of 5FU treatment in the present experimental condition; bone marrow cells from 5FU-treated and control C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice were injected into 200-rad–irradiated WBB6F$_1$-*W*/*W*^{v} mice. The recipient mice were divided into two groups; one group of mice was killed on day 8 and the other group on day 14. The 5FU treatment decreased the number of bone marrow cells from about 25 × 10^{6}/femur to about 2 × 10^{6}/femur but increased the day 14 to day 8 ratio of spleen colonies as reported by Hodgson and Bradley[14] and van Zant[16] (Table 1). Because hematopoietic stem cells that form day 14 colonies are considered to have more extensive proliferative potential than those that form day 8 colonies,[6] we used bone marrow cells of 5FU-treated mice throughout the present study.

Because we injected as few as 10^{3} 5FU-treated bone marrow cells in the next experiment, we confirmed the number of CFU-S in such a small number of 5FU-treated bone marrow cells of C57BL/6-*Pgk-1*^{b}/*Pgk-1*^{a} mice. As shown in Table 1, 10 × 10^{3} cells contained ~2.2 day 14 CFU-S and 2 × 10^{3} cells, ~0.4 day 14 CFU-S.

Table 1. Numbers of Day 8 and Day 14 Spleen Colonies in 200-rad–Irradiated WBB6F$_1$-*W/W^v* Mice After Injection of 5FU-Treated and Nontreated Bone Marrow Cells

5FU Treatment*	No. of Cells Injected (× 10^3)	No. of Colonies (Mean ± SE)† Day 8	Day 14	Ratio of Day 14/Day 8 Colonies
No	50	4.4 ± 1.7 (10)	4.8 ± 1.0 (13)	1.1
Yes	50	0.7 ± 0.3 (12)	12.6 ± 0.8 (16)	18
Yes	10	ND	2.2 ± 0.6 (11)	ND
Yes	2	ND	0.4 ± 0.1 (12)	ND

Abbreviation: ND, not determined.

*Bone marrow cells were obtained from C57BL/6–*Pgk-1^b/Pgk-1^a* mice that had been injected with 5FU (150 μg/g body weight) four days before sacrifice.

†The number of recipients is shown in parenthesis.

Various numbers of bone marrow cells from 5FU-treated C57BL/6–*Pgk-1^b/Pgk-1^a* mice were injected into 200-rad–irradiated WBB6F$_1$-*W/W^v* mice; the hemoglobin pattern of the recipients was examined at 4-week intervals. When the hemoglobin pattern of WBB6F$_1$-*W/W^v* recipient mice (*Hbb^d/Hbb^s*) changed to pure *Hbb^s/Hbb^s*, the PGK pattern of erythrocytes was also examined.

In total, 303 WBB6F$_1$-*W/W^v* mice survived at least 16 weeks after bone marrow transplantation; in eight WBB6F$_1$-*W/W^v* mice, a pure *Hbb^s/Hbb^s* hemoglobin pattern was detected only at one observation time (transient reconstitution in Table 2, Fig 1), whereas pure *Hbb^s/Hbb^s* pattern was maintained at least during 8 weeks (ie, three observation times) in 48 WBB6F$_1$-*W/W^v* mice (long-term reconstitution in Table 2, Fig 2).

In 8 WBB6F$_1$-*W/W^v* recipient mice that showed transient reconstitution, erythrocytes of five mice showed either A-type PGK or B-type PGK, whereas erythrocytes of the remaining three mice showed AB-type PGK at the time of transient reconstitution. When transient reconstitution was lost (ie, hemoglobin pattern returned to *Hbb^d/Hbb^s*), the PGK pattern of erythrocytes also returned to type B in all aforementioned eight WBB6F$_1$-*W/W^v* mice.

WBB6F$_1$-*W/W^v* mice that were reconstituted by bone marrow cells of C57BL/6–*Pgk-1^b/Pgk-1^a* mice for a long term were divided into three groups by using the PGK pattern of erythrocytes as a criterion. In group 1, erythrocytes showed either A- or B-type PGK throughout the time of reconstitution. The reconstitution continued until the end of the observation period (40 weeks) or death in six of seven mice that belonged to group 1 (Fig 2A). In the remaining one WBB6F$_1$-*W/W^v* mouse, the hemoglobin pattern came back to *Hbb^d/Hbb^s* 28 weeks after bone marrow transplantation (Fig 2B). In group 2, erythrocytes showed AB-type PGK when the change of hemoglobin pattern to pure *Hbb^s/Hbb^s* was first detected. Then, the PGK pattern changed to either type A or B (Fig 2C). In group 3, erythrocytes showed AB-type PGK throughout the time of reconstitution (Fig 2D). In some mice of group 3, the type A–to–type B ratio of PGK was not always constant but fluctuated from time to time (Fig 2D). The distribution of WBB6F$_1$-*W/W^v* recipient mice among the aforementioned three groups was apparently influenced by the number of injected bone marrow cells (Table 3). All WBB6F$_1$-*W/W^v* mice that received 10^3 or 3 × 10^3 bone marrow cells from 5FU-treated C57BL/6–*Pgk-1^b/Pgk-1^a* mice belonged to group 1, whereas all WBB6F$_1$-*W/W^v* mice that received ≧50 × 10^3 cells belonged to groups 2 or 3.

We calculated the concentration of stem cells that may reconstitute the whole erythropoiesis of WBB6F$_1$-*W/W^v* mice for a long term by using limiting-dilution analysis. From data shown in Table 2, we calculated the proportion of

Table 2. Number of WBB6F$_1$-*W/W^v* Mice (*Hbb^d/Hbb^s*) in Which a Transient or Long-term Change of Hemoglobin Pattern Was Observed After Injection of Various Numbers of Bone Marrow Cells From 5FU-Treated C57BL/6–*Pgk-1^b/Pgk-1^a* Mice (*Hbb^s/Hbb^s*)

No. of Cells (× 10^3)	No. of Mice	No. of Mice in Which Hemoglobin Pattern Changed (%) Transient	Long-term	Total
1	65	0 (0)	3 (5)	3 (5)
3	85	3 (3)	4 (5)	7 (8)
10	49	2 (4)	2 (4)	4 (8)
30	22	1 (5)	6 (26)	7 (32)
50	31	2 (6)	11 (36)	13 (42)
75	30	0 (0)	11 (37)	11 (37)
100	21	0 (0)	11 (52)	11 (52)

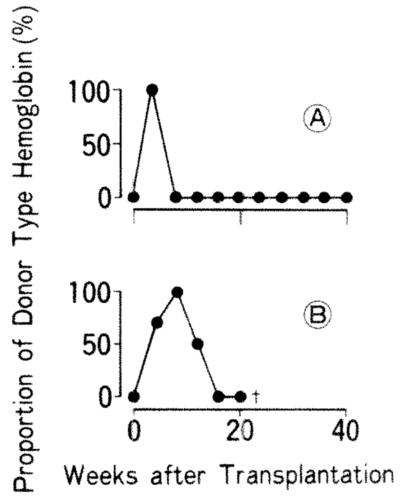

Fig 1. Typical cases of transient reconstitution. Electrophoretic pattern of hemoglobin of WBB6F$_1$-*W/W^v* mice (*Hbb^d/Hbb^s*) changed to the donor type (*Hbb^s/Hbb^s*) at 4 weeks (A) or 8 weeks (B) after the injection of bone marrow cells from 5FU-treated C57BL/6–*Pgk-1^b/Pgk-1^a* mice; 10 × 10^3 cells were injected in A and 3 × 10^3 cells in B.

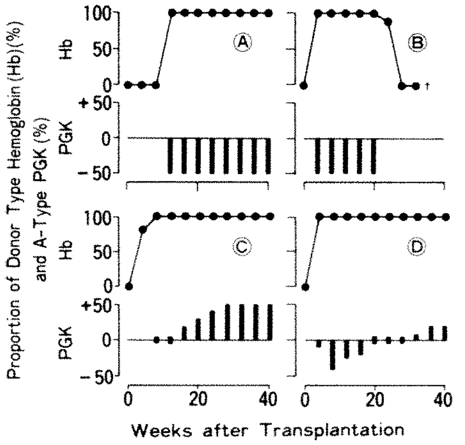

Fig 2. Typical cases of long-term reconstitution. Proportions of donor-type hemoglobin and A-type PGK are shown. The data of the proportion of A-type PGK for each mouse are plotted as the deviation of individual samples from the percentage of A-type PGK in C57BL/6–*Pgk-1*^{b}/*Pgk-1*^{a} mice according to Burton et al.[12] Therefore, +50% indicates that only A-type PGK is present, 0% that A-type PGK and B-type PGK were equally mixed, and −50% that only B-type PGK is present. (A) The WBB6F$_1$-*W*/*W*^{v} mouse was injected with 10^3 bone marrow cells from 5FU-treated C57BL/6–*Pgk-1*^{b}/*Pgk-1*^{a} mice. Erythrocytes showed B-type PGK alone throughout the observation periods (group 1). (B) The reconstitution ended at 28 weeks after the injection of 3 × 10^3 cells (group 1). (C) Erythrocytes showed both A-type and B-type PGK when hemoglobin first changed to the donor type at 8 weeks after the injection of 75 × 10^3 bone marrow cells. The PGK pattern changed to type A (group 2). (D) The WBB6F$_1$-*W*/*W*^{v} mouse received 50 × 10^3 bone marrow cells. Although erythrocytes showed both A-type and B-type PGK throughout the observation period, the proportion of A-type PGK fluctuated gradually (group 3).

WBB6F$_1$-*W*/*W*^{v} mice in which long-term complete reconstitution of erythropoiesis did not occur after bone marrow transplantation (proportion of unsuccessful transplantation), and plotted this against the number of injected cells (Fig 3). A straight line was obtained ($P < .01$ by χ^2 test) that intercepted the ordinate at 0.96. Therefore, the proportion of unsuccessful transplantations may be used to estimate the fraction of stem cells in 5FU-treated bone marrow cells. The slope of the line in Fig 3 is the estimate of the fraction of stem cells. Calculation of its 95% confidence limits was carried out according to the method described by Porter and Berry[25] and Breivik.[26] The concentration of such a stem cell that may reconstitute the whole erythropoiesis for a long term was 0.7 (95% confidence limits, 0.6 to 0.9) per 10^5 5FU-treated bone marrow cells.

Table 3. PGK Type of Erythrocytes in WBB6F$_1$-*W*/*W*^{v} Mice (*Hbb*^{d}/*Hbb*^{s}) in Which the Hemoglobin Pattern Changed to the Donor Type (*Hbb*^{s}/*Hbb*^{s}) for at Least 8 Weeks

No. of Bone Marrow Cells Injected (× 10^3)	No. of Mice at the Following PGK Type			
	Throughout A or Throughout B	AB to A or AB to B	Throughout AB	Total
1	3	0	0	3
3	4	0	0	4
10	0	0	2	2
30	1	0	5	6
50	0	6	5	11
75	0	6	5	11
100	0	4	7	11

This is the same experiment as shown in Table 2.

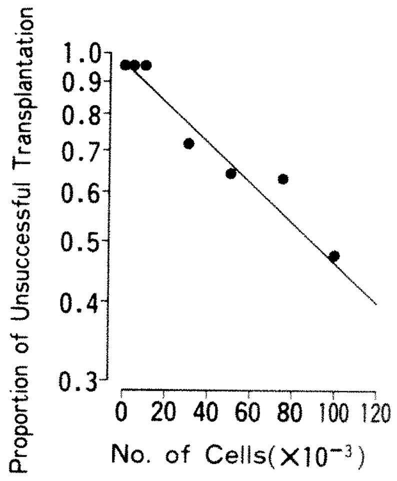

Fig 3. Proportion of WBB6F$_1$-*W*/*W*^{v} mice in which the hemoglobin pattern changed to the donor type for at least 8 weeks after the injection of various numbers of bone marrow cells from 5FU-treated C57BL/6–*Pgk-1*^{b}/*Pgk-1*^{a} mice. The logarithmic regression line is described by the equation $\ln P = -0.69 \times 10^{-5} x - 0.04$. The slope of this line gives an estimate of the fraction of stem cells with extensive self-maintaining potential among injected bone marrow cells.

DISCUSSION

When bone marrow cells of 5FU-treated C57BL/6–*Pgk-1*^{b}/*Pgk-1*^{a} mice were injected into 200-rad–irradiated WBB6F$_1$-*W*/*W*^{v} mice, the donor-type hemoglobin pattern (*Hbb*^{d}/*Hbb*^{s}) was detectable either transiently or for a long term (at least 8 weeks). Although the proportion of WBB6F$_1$-*W*/*W*^{v} mice with the long-term reconstitution increased in parallel with the number of injected bone marrow cells, transient reconstitution did not occur when ≧75 × 10^3 bone marrow cells were injected. Transient reconstitution probably results from lodgment and differentiation of stem cell(s) with limited proliferative potential, whereas the long-term reconstitution may be attributable to the successful transplantation of stem cell(s) with extensive proliferative potential. When comparably large numbers of bone marrow cells were injected, the probability that both types of stem cells were successfully transplanted might increase. In such cases, transient and long-term reconstitutions occurred at the same time and were recognized as long-term reconstitution.

The PGK pattern of erythrocytes in long-term reconstituted WBB6F$_1$-*W*/*W*^{v} mice was influenced by the number of injected bone marrow cells. The proportion of reconstituted mice with double enzyme patterns increased in parallel with the number of cells. When each WBB6F$_1$-*W*/*W*^{v} mouse received 10^3 or 3 × 10^3 bone marrow cells, erythrocytes of all seven long-term reconstituted WBB6F$_1$-*W*/*W*^{v} mice showed either an A-type or B-type PGK pattern (group 1 of Fig 2). The result appears to indicate that a single stem cell with extensive proliferative potential reconstitutes the whole erythropoiesis of these seven WBB6F$_1$-W/W^v mice for at least 8 weeks due to the following reason. In a population of stem cells consisting of equal numbers of A- and B-type cells, the probability that proliferation of *n* cells occurs in A-type cells only or in B-type cells only is $(1/2^n) \times 2$, ie, $1/2^{n-1}$. Consequently, the 100% (7/7) incidence of single-enzyme reconstitution cannot be explained in the case of $n \geqq 2$.

Estimation by limiting-dilution analysis also supports the possibility of monoclonal reconstitution. Because 10^3 bone marrow cells were injected into 65 WBB6F$_1$-W/W^v mice and 3×10^3 cells into 85 WBB6F$_1$-W/W^v mice, in total, 3.2×10^5 cells were injected into 150 WBB6F$_1$-W/W^v mice. The concentration of stem cells with an extensive proliferative potential was $0.7/10^5$ bone marrow cells, which indicated ~5 such stem cells in 3.2×10^5 bone marrow cells. When ~5 stem cells are injected into 150 WBB6F$_1$-W/W^v mice, the possibility that two stem cells are injected into a single WBB6F$_1$-W/W^v mouse should be low.

When calculated by using the concentration of day 14 CFU-S shown in Table 1, 3.2×10^5 bone marrow cells from 5FU-treated C57BL/6-$Pgk\text{-}1^b/Pgk\text{-}1^a$ mice have the capacity to produce about 64 to 81 day 14 spleen colonies in 200-rad–irradiated WBB6F$_1$-W/W^v mice. If a single day 14 CFU-S had been able to reconstitute the whole erythropoiesis of a WBB6F$_1$-W/W^v mouse, about a half of 150 WBB6F$_1$-W/W^v mice injected with 10^3 or 3×10^3 bone marrow cells would have shown a pure Hbb^s/Hbb^s hemoglobin pattern. In fact, three WBB6F$_1$-W/W^v mice showed a pure Hbb^s/Hbb^s pattern transiently and seven WBB6F$_1$-W/W^v mice for a long term. Therefore, most of the stem cells that produce a day 14 spleen colony do not appear to have the potential to reconstitute the whole erythropoiesis of a WBB6F$_1$-W/W^v mouse even transiently. This is consistent with the results of Wiktor-Jedrzejczak et al,[8] Boggs et al,[9] and Visser et al[27] that the CFU-S assay does not represent the number of stem cells with extensive proliferative potential.

Wiktor-Jedrzejczak et al,[8] Boggs et al,[9] and Visser et al[27] used bone marrow cells from nontreated syngeneic donors, whereas we harvested bone marrow cells from 5FU-treated donors of the parental strain origin. The number of bone marrow cells necessary to reconstitute a WBB6F$_1$-W/W^v mouse was smaller in their experiments than in our present experiment. Although 5FU treatment significantly increased the day 14–to–day 8 ratio of colonies, it does not appear to increase the proportion of stem cells with extensive proliferative potential. However, because we used C57BL/6-$Pgk\text{-}1^b/Pgk\text{-}1^a$ mice rather than syngeneic +/+ mice as donors, there is a possibility that the hybrid resistance reported by Harrison et al[20] in the present strain combination may reduce the efficiency of the stem cells of C57BL/6 strain origin.

Long-term reconstitution occurred in 33 of 82 WBB6F$_1$-W/W^v mice after transplantation of 50×10^3 to 100×10^3 bone marrow cells from 5FU-treated C57BL/6-$Pgk\text{-}1^b/Pgk\text{-}1^a$ mice. In all these 33 WBB6F$_1$-W/W^v mice, the PGK pattern of erythrocytes was of the AB type when the hemoglobin pattern first changed to the Hbb^s/Hbb^s type. The PGK pattern changed from a double-enzyme pattern to a single-enzyme pattern in 16 of 33 WBB6F$_1$-W/W^v mice (group 2 of Fig 2), but the double-enzyme pattern remained in 17 WBB6F$_1$-W/W^v mice (group 3 of Fig 2).

There are two possible explanations for group 2. (*a*) The reconstitution may result from the lodgment and differentiation of both a stem cell(s) with limited proliferative potential and a single stem cell with extensive proliferative potential. A stem cell with extensive proliferative potential continued the proliferation and differentiation after the expiration of stem cell(s) with limited proliferative potential. (*b*) The reconstitution may be initiated by lodgment and differentiation of two or more stem cells with extensive proliferative potential. However, subsequent emergence of a single monoclonal population occurred. Probably, stem cells belonging to the other clone(s) may remain in the hematopoietic tissues, but their differentiation was suppressed by unknown mechanisms. Determination of PGK patterns of CFU-S and in vitro colony-forming cells from such hematopoietic tissues may prove or disprove the possibilities.

In group 3, long-term reconstitution appears to be attributable to the lodgment of ≧2 stem cells with extensive proliferative potential. Even when the AB-type PGK pattern remained throughout the observation period, the type A–to–type B PGK ratio fluctuated in some WBB6F$_1$-W/W^v recipients. This is consistent with the recent result of Lemischka et al[28] and may be explained by the concept of clonal succession proposed by Kay.[29]

To investigate the fate of a single stem cell, Abramson et al[30] induced chromosomal abberations by x-irradiation, and Dick et al,[31] Lemischka et al,[28] and Keller et al[32] marked the stem cells with the integration sites of retrovirus. These markers are very useful because of the huge numbers of variations. However, when these markers are used, it seems difficult to exclude the influences of irradiation or retrovirus infection. Moreover, clones that failed to bear abnormal chromosomes or retrovirus and clones of the recipient origin are not detectable. These difficulties may be overcome with the present system in which electrophoretic patterns of hemoglobin and PGK are used as markers. Furthermore, vast numbers of recipient mice can be investigated frequently because it is much easier to examine patterns of hemoglobin and PGK than to examine chromosomal aberrations or integration sites of retrovirus. The present system is especially useful as a model of monoclonal reconstitution because a lot of information about human stem cell disorders such as CML, polycythemia vera, and agnogenic myeloid metaplasia has been accumulated by using another X chromosome–linked enzyme, G6PD, as a marker.[10]

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Analysis of the Erythroid Phenotype of HEL Cells: Clonal Variation and the Effect of Inducers

By Th. Papayannopoulou, B. Nakamoto, S. Kurachi, and R. Nelson

The erythroid phenotype of HEL cells, before and after the addition of a variety of inducers, was assessed at the cellular and biochemical level. Among 14 inducers used, δ-aminolevulinic acid (δ-ALA) was identified as the most optimal inducer of heme and globin synthesis in HEL cells. The relative synthesis of globin chains produced by HEL cells, mainly γ and α chains with traces of ϵ and ζ chains, was not influenced by the majority of the inducers used. However, δ-ALA and bromodeoxyuridine did increase the relative synthesis of α and ϵ chains respectively. Subcloning experiments revealed heterogeneity in the constitutive expression of α globin; however, the latter was inducible in all clones by either hemin or δ-ALA. One rare clone of HEL cells was found to produce, in contrast to parental cells, significant amounts of ϵ globin. This clone differed from K562 cells by the absence of any ζ globin expression, thus demonstrating the independent regulation of the two embryonic chains, ϵ and ζ. Changes in the expression of several surface markers specific for erythroid cells were found to accompany the globin accumulation in these cells, and some of these changes appeared to be inducer specific. Thus, the unique globin and nonglobin phenotypic properties of HEL cells and their subclones make them valuable cellular models complementary to the existing K562 cells for studying regulatory aspects of erythroid-specific proteins.

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ESTABLISHED ERYTHROLEUKEMIA cell lines capable of constitutive and induced globin expression are valuable tools for the investigation of cellular and molecular mechanisms regulating globin expression and erythroid differentiation. We have previously described the establishment of a human erythroleukemia line, the HEL cells,[1] which, like K562 cells,[2] are capable of human globin expression. Detailed characterization of the surface antigenic profile of HEL cells[3] disclosed that these cells display several erythroid-specific surface markers and, in addition, features of other nonerythroid cell lineages (ie, platelet/megakaryocytic lineage) that have been the subject of several recent studies.[4-12] However, only preliminary data have been presented to date on the erythroid phenotype of HEL cells.[1] In the present study, we report detailed biochemical characterization of globin produced by HEL cells before and after induction by several inducing agents. Furthermore, through the availability of antiglobin chain–specific monoclonal antibodies (MoAbs) we were able to study, at the cellular level in both HEL and K562 cells, the distribution of the various globins at basal conditions and its modulation by the inducers. The augmentation in globin expression was attended by certain coordinate changes in the surface erythroid phenotype of HEL cells, as assessed through the use of monoclonal antibodies with erythroid specificity. The present analysis and comparative studies with K562 cells indicate that these two lines, by virtue of their unique characteristics and their composite phenotype, complement each other as models to be exploited for studies on the regulation of globin expression.

MATERIALS AND METHODS

Cell Lines and Culture Methods

HEL cells have been in continuous culture in our laboratory since their establishment in 1980. Three different sublines of K562 cells were used for comparison: clone C-16, obtained from Dr M. Fukuda[13]; K562 cells from Dr J. Hansen (K562-H); and K562(S) cells from Dr G. Rovera.[14] Both K562 and HEL cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, pyruvate, and antibiotics in a humidified 5% CO_2 incubator. Cells were split and transferred into fresh medium at biweekly intervals. All cultures were tested periodically to ensure the absence of mycoplasma. To obtain cell doubling time in suspension cultures, daily cell counts in a hemocytometer were done. To derive clones from HEL or K562 cells, two systems of cloning were used: the limited-dilution method in microtiter wells or cloning in methylcellulose (MC) semisolid media and subcloning of individual clones initially lifted from MC plates. Cloning of HEL and K562 cells was also carried out in plasma clot cultures, but these cultures were used primarily for either benzidine staining or immunofluorescence staining of colonies (see the following sections).

Induction

During log-phase growth (about 2 × 10^5/cells/mL), a given inducer (Table1) was added at various doses to the culture media, and aliquots of cells were taken for testing at several intervals after the addition of inducer. The effect of various inducers on heme synthesis was assessed by benzidine staining and total hemoglobin determination, whereas the effect on globin was evaluated by globin chain analysis and by anti-globin immunofluorescence labeling. The effect on surface markers was evaluated by surface immunofluorescence labeling using antibodies with known specificities.

Benzidine Staining

For evaluation of heme or hemoglobin induction in HEL or K562 cells, benzidine staining was used in either cell suspension[15] or in fixed cytocentrifuge smears. For the latter, an adaptation of the method described by McLeod et al[16] was used.

Hemoglobin Analysis

Hemoglobin determination in lysates of induced and uninduced cells was carried out by a spectrophotometric method,[17] whereas separation of the hemoglobin species present was achieved by isoelectric focusing in polyacrylamide gels as described[18] with slight modifications. Hemoglobin bands were visualized by benzidine staining. For definitive identification of hemoglobin bands in our gels, we isolated their constituent chains (see the next section) in globin chain isoelectric focusing gels by using sliced bands from hemoglobin gels.

Globin Chain Analysis

For globin chain separation, lysates from ^{3}H-leucine–labeled cells (for an eight-12-hour period) were subjected to isoelectric focusing

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according to a method described by Rhighetti et al[19] and as adapted by us.[20] Before isoelectric focusing, globin was purified through haptoglobin binding.[21] Under our conditions of isoelectric focusing, all human chains (ϵ, ζ, γ, β, α) can be distinctly separated from each other. After isofocusing, the gels were fixed, treated with En^{3}Hance (DuPont, Boston), dried, and subjected to fluorography and densitometry as described.[20]

Immunofluorescence With Antiglobin Chain Antibodies

Cytocentrifuge smears prepared from uninduced and induced cells were fixed in methanol and reacted with antiglobin chain antibodies[22] in an indirect immunofluorescence assay using antimouse IgG (F(ab')$_2$) conjugated to fluorescein isothiocyanate (FITC) as a second antibody. Two MoAbs, one against γ chains and one specific for β chains, were used in a 1:1,000 dilution of ascites fluid. A polyclonal antibody monospecific for ζ chains was also used. This antibody was made by using Hb Portland as an immunogen, and it was kindly provided to us by Dr D. Chui.[23]

Surface Immunofluorescence

Several previously characterized MoAbs were used in an indirect immunofluorescence assay for evaluating the membrane reactivity of induced v uninduced cells. These antibodies include antiglycophorin A (R-10 and F-11),[3] EP-1 and EP-2,[24] anti-i (Den), anti-I (Ma),[25] anti-H (BE2 and *Ulex europaeus* I),[25] and antitransferrin receptor antibodies OKT9[26] and BE/25[27] as well as a recent series of antierythroid MoAbs generated by us.[28] Labeled cells were viewed under a Zeiss epifluorescence microscope through the appropriate filters, or they were subjected to fluorometric analysis in a fluorescence-activated cell sorter (Ortho system 50H Cytofluorograf with a 2150 computer, Westwood, MA).

Subcloning

Subcloning of HEL cells was done in MC media. Single colonies grown in MC were subcloned at least twice to ensure a single-cell origin of the clones. Subcloning of K562 cells was carried out by the limited-dilution method. After cloning, the isolated clones were expanded in suspension cultures, and they were induced and evaluated for globin expression and benzidine positivity, as indicated for parental cells.

Commitment Assay

To test whether a brief exposure to inducers leads to an irreversible decision for terminal differentiation in the absence of the inducer, a two-step commitment assay was performed as described previously for K562 cells[29] or MEL cells.[30] Briefly, after a one-day exposure of HEL and K562 cells to an optimal concentration of an inducer, the cells were washed to remove the inducer, counted, and plated (at 1,000 to 3,000 cells/mL) in Iscove's modified Dulbecco's medium (IMDM) or RPMI 1640, 30% fetal calf serum (FCS), and 0.8% MC. Colony number as well as the proportion of benzidine-positive colonies[30] was determined at day 5 postplating.

RESULTS

Induction of Heme Synthesis

Uninduced HEL cells showed only occasional benzidine-positive cells (Table 1), and hemoglobin levels in these cells were between 0.2 and 0.4 pg hemoglobin/cell. The effect of the various inducers on benzidine postivity is shown in Table 1. The addition of δ-aminolevulinic acid (δ-ALA) to growing HEL cells yielded the most optimum induction, as assessed by benzidine positivity and hemoglobin analysis; up to one third of the cells became benzidine-positive, and the total hemoglobin content increased from three- to tenfold, ie, from 0.4 to 3 to 4 pg hemoglobin/cell. Most of the other inducers caused a slight increase in benzidine positivity and, in contrast to δ-ALA, had an early adverse effect on the proliferation and viability of HEL cells. The metalloporphyrins (Zn-protoporphyrin-IX, Co-protoporphyrin IX, or protoporphyrin-IX) were very toxic to HEL cells, which resulted in significant cell death during the first three or four days in culture. Hemin solutions and hemolysates, however, were much less toxic. It is notable, however, that HEL cells are far more sensitive than K562 cells to the toxic effects of hemin. The effect of hemin on new heme synthesis could not be assessed with confidence when using the wet benzidine stain because of nonspecific positivity, presumably due to the uptake of exogenous hemin. Among the various HEL clones tested, including an earlier passage of HEL cells (HEL-92), there was no significant variability in benzidine positivity, either before or after induction. However, one clone, HEL-R, showed significant levels of benzidine positivity, especially after induction (Table 2) and, in that respect, differed from the other parental HEL cells or subclones tested.

Apart from the presence of the specific inducer, the level and type of serum used seemed to influence the level of induction. The induction potentiating property of the sera tested was as follows: 20% FCS > 10% FCS > newborn bovine serum > human serum > horse serum.

Induction of Globin Synthesis

It was previously shown that the main globin species produced by uninduced HEL cells is Bart's hemoglobin (Fig 1). Only traces of hemoglobin fractions comigrating with embryonic hemoglobins (Gower I, Gower II) or with hemoglobin F were present. This phenotype was confirmed by determining the chain composition of the major hemoglobin fractions in two-dimensional gels. This type of analysis disclosed that the fraction migrating in the position of hemoglobin Portland is composed not of γ and ζ chains but of a mixture of acetylated and nonacetylated Gγ and Aγ chains (Fig 1). Furthermore, as expected from hemoglobin analysis, globin chain isofocusing of ^{3}H-leucine–labeled lysates showed either trace or undetectable levels of ϵ and ζ chains and a variable amount of α chains. Although the major globin species are γ chains, the proportion of Gγ to Aγ is not as in fetuses because there is an excess of Aγ chains instead of Gγ. The pattern of globin chain synthesis in HEL cells contrasts with the one present in K562 cells in which there is predominance of Gγ chains and abundant synthesis of embryonic globin chains (Figs 3-5).

At a cellular level, a small but significant proportion of uninduced HEL cells was labeled with anti-γ chain monoclonal antibody, whereas only rare cells were found to be positive with a polyclonal and monospecific antibody for ζ chains (Fig 2A). In no instance were β-positive cells seen. An antibody to ϵ chains was not available to assess the cellular distribution of ϵ globin. The effect of the various inducers on globin synthesis is shown in Table 1 and Figs 3 to 5. γ chain

Table 1. Effect of Different Inducers on the Benzidine and γ-Globin Positivity of HEL Cells

Agents	Concentration	Days of Treatment	No. of Cells (Percentage of Control)	Viability (%)	Benzidine+ (%)	γ Globin+ (%)
None			100	>90	<1.0	3-10*
Protoporphyrin IX	50 μmol/L	7	26	60	0.4	<1.0
Co-Protoporphyrin-IX	50 μmol/L	5	49	83	0.5	<2.0
		10	13	52	—	—
Zn-Protoporphyrin-IX	25 μmol/L	3	64	95	—	—
		6	5	10	—	—
Hemin	50 μmol/L	7	90	90	(96)	20-50
Hemolysate	0.45 gHb/dL	3	100	95	2.2	10-30
δ-ALA	500 μmol/L	3	100	94	<1-2.0	40-60
		7	100	92	5-20	60-80
Hydroxyurea	6.57×10^{-5} mol/L	3	75	84	0.24	<1.0
				88	0.0	
DMSO	0.5%	3	—	63-97	0.8	Rare
		6	—	77	0.3	
Sodium butyrate	1 mmol/L	3	50	14	0.4	1-5
	0.5 mmol/L	3	100	88	1.1	39.0
Bleomycin	2.4×10^{-6} mol/L	3	33	59	1.4	10-15
		6	50	60	0.8	
Ara-C	3.5×10^{-7} mol/L	3	48	49	0.6	—
	1×10^{-8} mol/L	6	16	7	—	—
	5.0×10^{-5} mol/L	4	88	96	5.0	—
BrDU	3.25×10^{-5} mol/L	4	—	34	0.2	15-21
	3.25×10^{-5} mol/L	3	58	92	1.3	39.8
	1.62×10^{-5} mol/L	3	88	91	2.0	40.3
Actinomycin D	1.5×10^{-9} mol/L	3	91	90	0.1	6
		12	—	93	1.0	18
L-Ethionine	1 mmol/L	3	100	93	0.6	36.0

Abbreviations: DMSO, dimethyl sulfoxide; Ara-C, cytosine arabinoside; BrDU, bromodeoxyuridine.

*When a range is given, it indicates values from multiple experiments.

positivity by immunofluorescence was used as a screening of globin induction with all inducers, and labeling of cell lysates with ^{3}H-leucine was carried out only in the most potent inducers identified by the screening assay. The majority of the inducers appeared to somewhat augment γ globin synthesis. However, only few of them appeared to influence the specific globin pattern of HEL cells. Thus, hemin and δ-ALA consistently increased the proportion of α-chains, as described previously for K562 cells,[31] whereas 5-azacytidine and BrDU increased the relative expression of ϵ chains. The effect on ϵ globin was mostly evident in a subclone of K562 cells, C-16, which does not synthesize significant levels of ϵ chains in the uninduced state (Fig 3). Also, the addition of BrDU and sodium butyrate increase the relative synthesis of ϵ globin in HEL-92, whereas δ-ALA did not (Fig 5 and data not shown). Of interest, the addition of BrDU in HEL cells but not in K562 cells induced the appearance of a significant proportion of adherent, elongated cells (data not shown). Because of the demonstrated cellular heterogeneity in ζ globin and a presumed similar heterogeneity in ϵ globin expression, subcloning of HEL cells was carried out in the hope of obtaining globin phenotypes that differed significantly from the parental cells. Among a total of 20 clones tested in detail, the only variation was the one concerning the α chains (data not shown). However, one HEL clone obtained from parental HEL cells repeatedly treated for mycoplasma contamination was found to be distinctly different from the parental population. This subclone, designated HEL-R, is characterized by a high level of ϵ chain expression and higher levels of α chain expression compared

Table 2. Cellular Distribution of Individual Globins in HEL and K562 Cells Before and After Induction

	Benzidine+ Cells (%)			γ Globin+ Cells (%)			ζ Globin+ Cells (%)			β Globin+ Cells (%)		
Inducer*	K562(S)	HEL-92	HEL-R	K562(S)	HEL-92	HEL-R	K562(S)	HEL-92	HEL-R	K562(S)	HEL-92	HEL-R
None	30	0-1†	3-6	34.0	3-9	13-28	15-45	<1.0	0	0.4	0	0
Hemin	(97)	(96)	—	70	35	—	35	2.0	—	Rare	0	—
δ-ALA	93	24	43.0	81	34-67	68-91	27-55	2.5	0	2-4	0	0
5-Azacytidine	51	<1.0	—	61	10	—	48	—	—	1.5	—	—
Butyrate	42	1.1	4.0	77	<5	41.0	47	—	0	1.5	0	0
Ara-C	—	12	7.0	—	34	36.0	—	—	0	—	0	0
BrDU	—	1.3	8.0	18.5	39	49.0	18	—	0	1.3	0	0

*Cells tested four to six days after treatment.

†Ranges indicate values from several independent experiments.

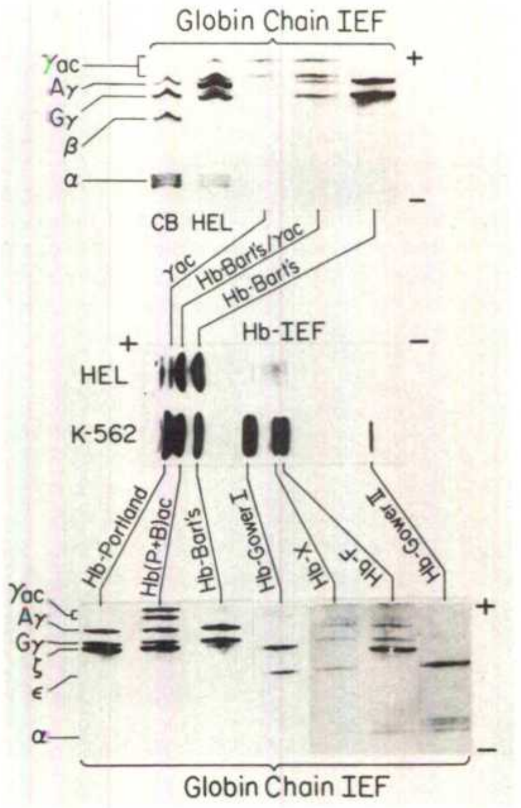

Fig 1. Hemoglobin (Hb) and globin chain isoelectric focusing of K562 (K562-H) and HEL lysates (^{3}H-leucine labeled). Individual hemoglobin species separated in hemoglobin isoelectric focusing gels (Hb-IEF) were sliced and run in noxidet P40-urea gels to analyze their constituent globin chains (globin chain IEF gels). Major Hb bands from HEL cells are Bart's and Bart's acetylated (B-ac), which migrates in the position of Hb Portland. Embryonic globins (Gower I, Gower II, Hb-X) as well as Hb F are minor bands in this HEL subline (severely α chain deficient), although there were considerably more in HEL-R. Hb-IEF, first-dimension Hb gels stained with benzidine; globin chain IEF, second-dimension gels showing radiolabeled globin chain bands; CB, Cord blood lysate; γac, acetylated γ chains.

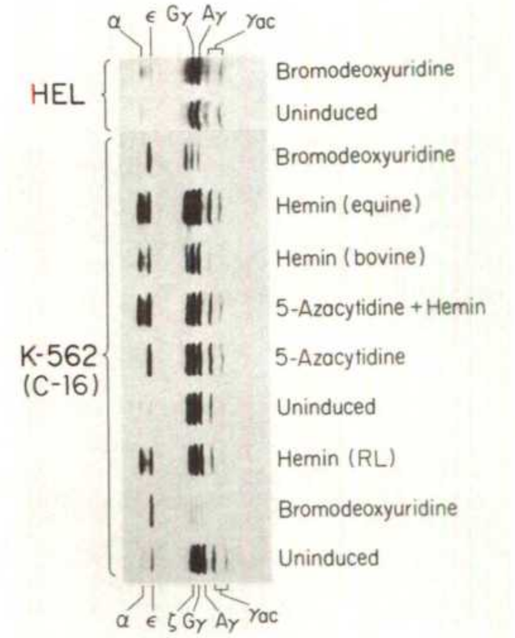

Fig 3. Effect of various inducers on the relative expression of globin chains in K562 (C-16) and HEL cells. Note the increase in α chains by hemin and the relative increase in ϵ chains by 5-azacytidine and BrDU. (Hemin [RL] is hemin crystallized by Dr R. Labbe', University of Washington.)

with parental cells (Fig 5). However, ζ chain is virtually absent, and this feature as well as the increased proportion of Aγ over Gγ chains distinguishes this subclone from K562 cells (Fig 5). In addition to the high levels of ϵ globin production, this clone was characterized by high constitutive levels of heme and total globin synthesis (ie, higher basal levels of γ globin and benzidine-positive cells) and by its high inducibility by the same inducers used for parental cells (Table 2).

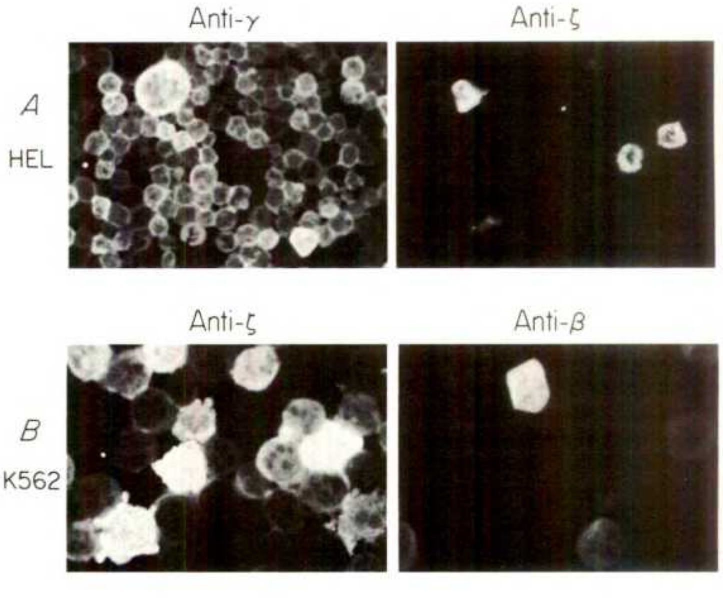

Fig 2. (A) Immunofluorescent labeling of δ-ALA-induced HEL cells by monoclonal anti-γ chain antibody conjugated to FITC and by monospecific anti-ζ antibody plus antirabbit IgG-FITC. Preparations are of similar cellular densities and photographed at the same magnification (×312). Note the high proportion of γ+ cells and the very low proportion of ζ+ cells within this HEL population. (B) Immunofluorescent labeling of δ-ALA-induced K562 (C-16) cells with anti-ζ antibody and with monoclonal anti-βδ antibody conjugated to FITC. Although γ globin can be detected in the great majority of induced K562 (C-16) cells (see Table 2), a smaller proportion of cells is ζ-positive, and rare cells are βδ-positive. This positivity is attributable to the presence of δ rather than β chains (see text).

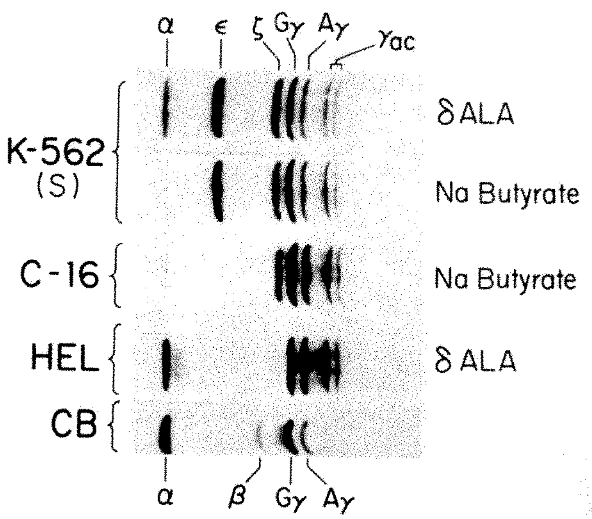

Fig 4. Globin chain isofocusing of ^{3}H-leucine-labeled K562(S) and K562 (C-16) cells induced by δ-ALA and sodium butyrate and of HEL cells induced by δ-ALA. Note the difference in ε chain expression between K562(S) and C-16 induced by the same inducer (butyrate) and the α chain increase by δ-ALA in both K562 and HEL (compare with uninduced cells in Fig 3).

Commitment to Terminal Maturation

The presence of γ globin-positive cells among the uninduced HEL cells and the rare benzidine-positive cells suggests that a certain proportion of HEL cells tends to accumulate higher hemoglobin as if they spontaneously undergo terminal maturation. To test whether spontaneous commitment to terminal maturation can also be observed or enhanced under conditions of clonal growth, we evaluated the number of benzidine-positive clones in a clonal assay. HEL cells were cloned in MC media, and colonies were evaluated six to ten days postplating. The morphology of colonies generated from HEL cells was characteristic and differed from the ones observed in K562 cells cultured for comparison. K562 cells produce compact and round colonies, but only one third of HEL colonies were of this type, the rest being diffused or scattered and reminiscent in morphology of human granulocytic-macrophage colonies. Subcloning of compact colonies tended to generate mostly compact colonies, and the same was true for noncompact colonies (data not shown). Spontaneous maturation was assessed by the appearance of a red color and by benzidine positivity of colonies in situ. There were no fully hemoglobinized (red) colonies from HEL cells, but colonies with benzidine-positive cells were up to 4%. In K562 plates, there were rare, small colonies with a reddish color and many more colonies with benzidine positivity. Colonies with apparent spontaneous maturation (any appearance of reddish color) were plucked from MC plates and labeled with tritiated leucine, and their lysates were used for globin chain isoelectric focusing. The globin phenotype of these spontaneously maturing HEL or K562 colonies was similar to the ones obtained in subclones (see the previous section). Of note, no β chains were again present in any of these spontaneously maturing colonies. To test whether commitment to terminal differentiation/maturation can be influenced by inducers, selective inducers were tested in a commitment assay as described before.[29] In preliminary experiments, we have determined that 24-hour exposure to the inducer followed by plating in MC in the absence of the inducer and by evaluation of colonies at day 5 are adequate conditions to detect any influence in commitment decision. Parallel experiments with K562 cells under these conditions were also carried out. The data obtained are shown in Table 3. An increase in the uniformly benzidine-positive colonies and in the mixed benzidine-positive colonies in K562 cells by these inducers was confirmed with these experiments. However, in HEL cells, at best, partial commitment by the same inducers, as judged by the assay used, could be observed. Of interest, virtually all benzidine-positive colonies in HEL cells were of the mixed type, which indicated that only part of the progeny becomes terminally committed.

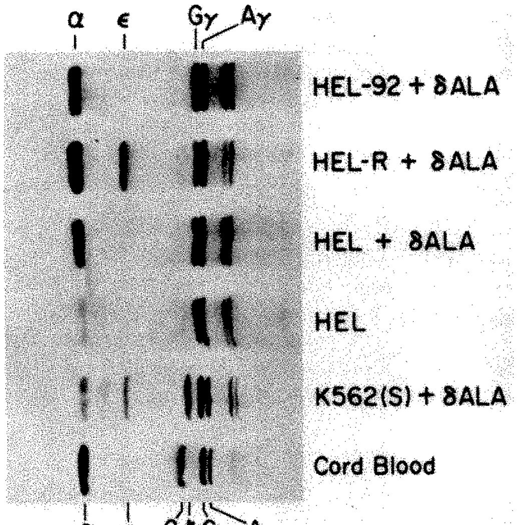

Fig 5. Isoelectric focusing of uninduced and induced ^{3}H-leucine-labeled cell lysates from HEL and HEL-R. The features that distinguish this clone from the parental line are the presence of high levels of ε chain, the increased levels of α chains, and its higher overall heme and globin inducibility.

Induction of Erythroid Surface Antigens

A detailed characterization of the membrane antigenic profile of HEL cells and its comparison with K562 cells has been previously presented.[3] It was also shown previously that treatment of HEL cells with 12-0-tetradecanoylphorbol-

Table 3. Induction of Commitment to Terminal Maturation

	Benzidine+ Colonies (%) After a 24-h Exposure to			
Cell Line	No Inducer	δ-ALA (0.5 mmol/L)	Sodium Butyrate (0.5 mmol/L)	Ara-C (5 × 10^{-8} mol/L)
K562(S)	29.5*	32.8	52.0	46.5
HEL-R	6.8	31.5	20.8	12.0
HEL-92	2.9	11.6	13.6	5.0

*Mean of two replicate MC plates counted on day 5 postplating.

13-acetate (TPA) reduced the abundance of some of the erythroid markers (ie, glycophorin, globin) while, at the same time, the cells changed their behavior and became noninducible.[4] In this study, we examined whether globin induction in HEL cells was accompanied by changes in their surface erythroid antigens.

Glycophorin. Uninduced and δ-ALA–induced HEL cells were treated with two MoAbs against glycophorin, R-10, and F-11 and then with antimouse $F(ab')_2$-IgG (or IgM) conjugated to FITC and subjected to analysis (Fig 6). Before induction, as previously noted,[3] virtually all cells were positive with R-10. However, induction by δ-ALA resulted in a rightward shift of the profile toward greater intensity of fluorescence. Although this shift was reproducible, the magnitude of the change was not great. In contrast to findings with R-10, a significant change was observed with antibody F-11. The latter, although highly reactive with normal erythroblasts,[3] shows a very low intensity of fluorescence in uninduced cells; however, after induction, a significant shift toward higher fluorescence intensity is found (Fig 6). Similar changes were produced with hemin induction, but the changes were greater and more reproducible with δ-ALA induction.

iI-antigens. HEL cells expressed i antigen, and only an occasional cell reacted with anti I serum.[5] The behavior of iI antigenic expression was tested after induction of HEL cells with hemin, δ-ALA, sodium butyrate, and BrDU. The last two inducers were chosen because of their reported influence on I antigen expression in K562 cells.[32] Hemin and δ-ALA caused only minor changes in I expression. However, BrDU and butyrate increased the expression of I antigen (Fig 7). Because these two agents, ie, sodium butyrate and BrDU, cause a significant reduction in the proliferation of the cells at the doses used, we examined whether changes in the proliferative status of the cells could be responsible for the effect. To control for proliferation inhibition, we cultured HEL cells in media containing antitransferrin for six days. Such cells, as independent experiments showed, failed to proliferate, but they remained viable for that period of time. As seen in Fig 7, antitransferrin-treated HEL cells showed an increased expression of I antigen. Therefore, it is very likely that, at least in part, the increase in I antigen expression after sodium butyrate and BrDU is due to changes in their proliferative status, although a direct induction, especially by BrDU (Fig 7), cannot be entirely excluded. The interpretation of these changes is somewhat supported by the information that anti-I–positive K562 cells (separated by a cell sorter) showed an increased doubling time compared with anti-I–negative cells (data not shown). In addition, it is of interest that another unrelated antigen, BB-2, which is dramatically increased with butyrate treatments, is also enhanced upon the cessation of proliferation of HEL cells by the addition of antitransferrin or TPA.[4]

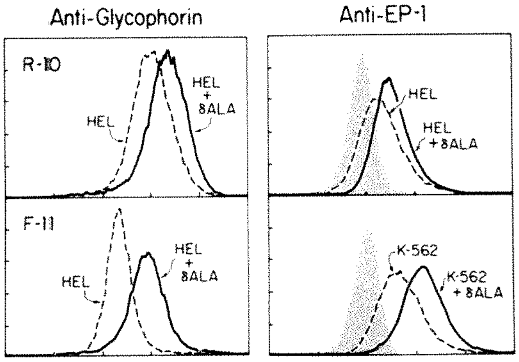

Fig 6. **HEL cells labeled with antiglycophorin MoAbs (R-10 and F-11) before and after induction by δ-ALA. Before induction significant reactivity with MoAb R-10 and much less so with F-11 was observed. After induction, however, reactivity with both MoAbs, especially with F-11, was increased, as indicated by a rightward shift in fluorescence intensity. Enhancement of expression of Ep-1 antigen in HEL and K562 cells after δ-ALA induction. Dotted peaks are negative control samples labeled with an irrelevant first antibody of the same idiotype as Ep-1 and with the same second antibody (x-axis, relative cell number; y-axis, fluorescence intensity).**

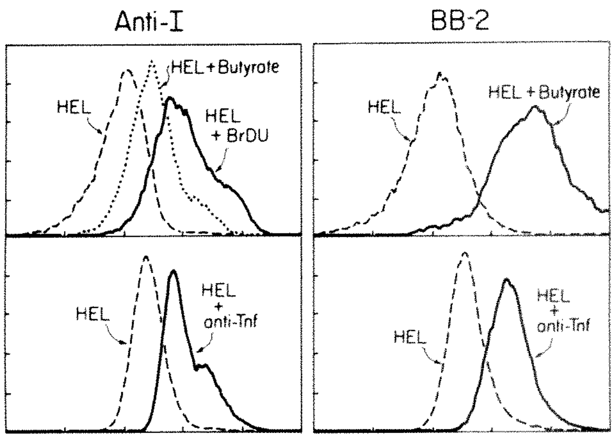

Fig 7. **Enhancement of expression of I antigen in HEL cells after induction by butyrate and BrDU. Both inducers yielded a weak induction of HEL cells, and the isolated I antigen increase is partly attributable to cessation of cell proliferation as indicated by control nondividing cells (HEL cells with antitransferrin) and by parallel changes in another antigen (present in all myeloid and B lymphoid cells) detected by antibody BB-2 (x- and y-axes are as in Fig 6).**

H antigen. The great majority of HEL cells are positively labeled with anti-H MoAb BE2[25] and with the lectin *Ulex europaeus* I. No significant changes were observed with induction.

Other erythroid surface antigens. We have recently described the characterization of two antierythroid-specific antibodies (anti–EP-1 and anti–EP-2) raised through the use of normal erythroid cells as immunogens.[24] Using these antibodies in K562 cells, we observed that their reactivity is increased after globin induction.[24] Similar experiments with δ-ALA–induced HEL cells have shown that this enhancement can also be observed in HEL cells after induction (Fig 6). Furthermore, we have tested the reactivity of induced HEL cells with a set of four new antierythroid monoclonals.[28] Augmentation in reactivity was again observed during the first three days of induction (data not shown).

Transferrin receptor. The increase in transferrin-bound iron in the developing erythroid cell correlates with the stages of the most active hemoglobin synthesis. We have examined the reactivity of uninduced and induced HEL or K562 cells with two antitransferrin receptor antibodies (OKT9 and BE/25). The data indicate that some increase in the expression or the density of transferrin receptors occurs after δ-ALA induction of either K562 or HEL cells. Because the cells' proliferation rate (doubling times) early on is similar to that of control cells, the increase in antitransferrin receptor reactivity may reflect an increase in the traffic of iron into the cells during induction.

DISCUSSION

Analysis of the basal and inducible erythroid phenotype of HEL cells performed in this study disclosed several points of interest. Among several inducers used, the effectiveness of δ-ALA was surprising. Augmentation in hemoglobin synthesis after δ-ALA addition was observed in the absence of any initial effects on the proliferative capacity and cell viability. Other inducers showed fewer effects on the augmentation of hemoglobin synthesis, and this was accompanied by more toxicity (cessation of proliferation and cell viability). It was of interest that δ-ALA was also a potent inducer in K562 cells. This remarkable responsiveness of these two human cell lines to δ-ALA addition may indicate that in both lines early steps in heme biosynthesis are rate limiting. Furthermore, because δ-ALA is not among the potent inducers of murine erythroleukemia cells,[33,34] biochemical differences within the heme synthetic pathway between the human and murine lines[35] may underlie these effects.

Studies on the cellular distribution of globin chains and the subcloning experiments suggested that, in contrast to the widespread expression of γ globin, expression of embryonic globins (ζ and ε) is restricted to a subpopulation of cells. This population, however, does not seem to synthesize exclusively embryonic globins and seems to overlap with cells expressing γ globin. The numerical relationships of γ-positive cells (over 90%) and ζ-positive cells (≃25%) in K562 cells, the double (γ and ζ) labeling experiments in the same cells (data not shown), and the significant production of ε globin by a population virtually all positive in γ globin—HEL-R and K562(S)—support this notion. This is of interest in view of previous evidence which, based on globin biosynthetic studies, showed that ζ globin expression is confined only to the embryonic cell population during normal development.[36] Subsequent studies, however, indicated that cells coexpressing fetal (γ) and embryonic (ε and ζ) globin do exist during the transitional period of embryonic to fetal erythropoiesis.[37,38] Thus, coexpression of fetal and embryonic globins in HEL-R and K562 cells may not be necessarily aberrant but a reflection of earlier developmental phenotypes realized through their leukemic transformation. Furthermore, the globin phenotype of HEL-R expressing high levels of ε in the virtual absence of ζ globin suggests that the two embryonic globins are independently regulated and provides a cellular model for further understanding of control mechanisms.

Expression of adult globins is virtually absent in both HEL and K562 cells although, in one study, a small number of δ and β mRNA copies were detected[39] and, in another, an unsubstantiated claim about hemoglobin A synthesis is made.[40] The small number of β-positive cells found in K562 cells in the present study is of interest. These cells were detected only by a MoAb reacting both with β and δ chains,[22] and they were not found in subsequent studies in which an anti-β chain antibody that does not crossreact with δ chains was used (kindly provided by Dr Jensen).[41] The presence of δ globin but not β globin, as implied by these studies, and the readily detected δ mRNA by previous studies[39,42] seem to suggest that either the factors for δ expression are different from those required for β expression[43] or that *cis* inhibition of β globin may be operative in these cells.

Induction of hemoglobin synthesis was, with few exceptions, not accompanied by changes in the specific types of globin. Some consistent trends, however, were noted (ie, preferential increase in α or ε chains), and these were shared by both HEL and K562 cells. Previously noted variations in the proportion of various hemoglobins or globins in K562 cells[40,44] can be easily reconciled by the findings presented here, ie, the heterogeneity in α and ε production by individual clones and the fact that some of the inducers favor the production of some chains over the rest.

Although globin is augmented after induction of HEL and K562 cells, a well-coordinated expression of the terminal maturation protein program, as it occurs in murine erythroleukemic cells, does not take place. Thus, minimum or no changes in nonglobin proteins have been pointed out in previous studies of K562 cells,[45] and the overall protein profile of induced cells does not resemble the protein profile of normal nucleated erythroid cells.[6] Nevertheless, despite the absence of a coordinated expression of the entire erythroid maturation program upon induction, a few nonglobin changes were noted, and these most likely are indicative of an attempt for further maturation; thus, glycophorin attains a form presumably more enriched in carbohydrate groups (Fig 6A), and the expression of some erythroid-specific antigens, recognized by MoAbs, is enhanced (Fig 6B). In addition, an increase in I antigen expression was noted with two inducers, BrDU and butyrate (Fig 7), as previous studies with K562 cells have pointed out.[32] It is of interest that, in our studies, increased I expression was also correlated with decreased proliferative activity and a decreased number of divisions in both HEL and K562 cells. However, the relevance of this finding to physiological increments in I antigen expression during erythroblast maturation[46] is unclear.

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Human Granulocyte-Macrophage Progenitors and Their Sensitivity to Cytotoxins: Analysis by Limiting Dilution

By Kathryn E. Barber, Philip S. Crosier, Steven Gillis, and James D. Watson

Limiting dilution analysis of granulocyte-macrophage progenitor cells was performed by using adherent and T cell-depleted normal human bone marrow and the recombinant human growth factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Estimated frequencies for progenitor cells responding to G-CSF were one in 489 for colonies scored at day 7, and one in 1,015 for day 14 colonies. For GM-CSF the frequencies were one in 1,407 (day 7) and one in 574 (day 14). The effects of tumor necrosis factor (TNF) and lymphotoxin (LT) on the frequency of progenitors responding to either G-CSF or GM-CSF was determined. Both TNF and LT inhibited the response of cells to G-CSF, and in these cultures the frequency of progenitor cells that responded to G-CSF was reduced to less than one in 100,000 cells. In contrast, the frequency of cells able to form colonies in cultures stimulated with GM-CSF was unaltered by either cytotoxin. This differential sensitivity to cytotoxins suggests that either G-CSF and GM-CSF are acting on separate granulocyte progenitor populations or that TNF and LT selectively influence the biochemical pathways associated with the activation of receptors for G-CSF.

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THE CYTOTOXINS tumor necrosis factor (TNF) and lymphotoxin (LT) are capable of exerting a number of effects on hematopoiesis in vitro.[1-7] Although TNF and LT are distinct molecular species with different antigenic determinants, there are clear similarities in both structure and biologic properties. The cDNA molecules for human TNF and LT have been isolated and expressed.[8-10] The recombinant products share 28% amino acid homology and 46% nucleotide homology, with areas of homology grouped predominantly in two regions of the molecules.[8] These homologous regions are likely to be important in specific receptor binding, and there is evidence that TNF and LT bind to the same cell surface receptor.[11]

We have recently reported that the suppressive effects of these molecules on granulocyte-macrophage progenitor cells are variable and dependent upon the growth factor used to stimulate granulocyte-macrophage colony formation. Colonies formed after stimulation with granulocyte colony-stimulating factor (G-CSF) are more sensitive to the suppressive effects of TNF and LT than are colonies formed after stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF).[12] This observation suggests that progenitors responsive to G-CSF are more sensitive to cytotoxin-induced inhibition than are those progenitors responsive to GM-CSF. To determine the frequency of human bone marrow progenitors sensitive to the effects of TNF or LT a limiting dilution analysis was performed. Cultures were stimulated with either recombinant human G-CSF or recombinant human GM-CSF. We report a striking difference between the number of progenitors responsive to G-CSF when no cytotoxin is present and the number capable of proliferative response to G-CSF in the presence of either TNF or LT. This is in marked contrast to cultures stimulated with GM-CSF where neither TNF nor LT influenced the progenitor cell frequency. These findings are important in determining the mode of action of each of these hematopoietic growth factors.

MATERIALS AND METHODS

G-CSF, GM-CSF, TNF, and LT. Recombinant human G-CSF (specific activity, 10^7 U/mg) and recombinant human GM-CSF (specific activity, 3.7×10^7 U/mg) were supplied by Immunex Corp, Seattle. Recombinant human TNF (specific activity, 5.02×10^7 U/mg) and recombinant human LT (specific activity, 1.2×10^8 U/mg) were generous gifts from Genentech Inc, South San Francisco.

Human bone marrow. Human bone marrow was obtained by aspiration from a panel of healthy volunteers. This procedure had hospital ethical committee approval. The marrow was prepared for use in limiting dilution assays by dilution in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with asparagine, benzylpenicillin, and streptomycin sulfate, which was followed by separation on Ficoll-Hypaque (density, 1.077 g/mL; Sigma Chemical Co, St Louis). Adherent cells were depleted by incubating the marrow cells in culture medium with 7.5% fetal calf serum (FCS, GIBCO) for 60 minutes at 37°C in 36 × 12-mm polystyrene dishes (10^7 cells/2 mL). Depletion of T cells was achieved by panning where nonadherent cells were incubated with optimal concentrations of Leu-1 and Leu-5 (Becton Dickinson, Mountain View, CA), UCHT1 (P. Beverley), and OKT4 and OKT8 (Ortho Pharmaceutical Corp, Raritan, NJ) for 20 minutes at 4°C; washed; resuspended at 2×10^7 cells/3 mL; and incubated for 70 minutes at 4°C on 100 × 15-mm polystyrene dishes coated with goat antibody to mouse immunoglobulin (Tago Diagnostics, Inc, Burlingame, CA). The nonadherent cells were recovered by swirling the plates and gently washing five times. The number of T cells contaminating the final preparation was assessed by incubation with goat antimouse IgG-fluorescein isothiocyanate (FITC) (Tago) for 20 minutes at 4°C then analysis by FACS 440 (Becton Dickinson). The number of T cells was always $<1\%$.

Limiting dilution assays. Limiting dilution assays were performed in 96-well microtitre plates (Nunc, Kamstrup, Denmark). Bone marrow cells were suspended in IMDM containing 0.3% agar,

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(Difco, Detroit), 20% FCS (vol/vol preselected batch; Gibco), and appropriate concentrations of growth factors and/or cytotoxins. G-CSF and GM-CSF were added at final concentrations of 100 U/mL, which are saturating for growth. TNF and LT were added at final concentrations of 100 U/mL, doses that have been shown to cause suppression of granulocyte-macrophage colony formation.[12] Cell concentrations ranged from 2.5×10^2/mL to 5×10^5/mL. Aliquots of 200 μL were plated in 24 microwells for each cell concentration. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 14 days. The number of wells containing one or more colonies (aggregates of 40 or more cells) were assessed at days 7 and 14.

Calculation of progenitor frequencies. Microcultures were scored as positive if they contained one or more colony. The percentage of nonresponding cultures was plotted against the responder cell number. The frequency of responding cells was calculated from experiments in which eight to 11 different doses of bone marrow cells were cultured by the reiterative, maximum-likelihood method.[13]

RESULTS

Frequency of granulocyte-macrophage progenitors resistant to the effects of cytotoxins. The effects of TNF and LT on the frequency of progenitor cells that responded to G-CSF and GM-CSF were examined. In these limiting dilution experiments cultures were stimulated with either G-CSF or GM-CSF, and for each growth factor, cultures contained either no cytotoxin, TNF, or LT. Cultures were scored at days 7 and 14. Progenitor cell frequencies were calculated for each combination of growth factor and cytotoxin at both time points.

The data presented in Fig 1 are from an experiment where adherent and T cell–depleted bone marrow was stimulated with either G-CSF (panels A and B) or GM-CSF (panels C and D). Panels A and C show the results of assays scored at day 7 and panels B and D, the results of assays scored at day 14. Estimates of progenitor frequency were calculated by maximum-likelihood determinations for each growth factor/cytotoxin combination and are presented in the legend. Similar results were obtained in two limiting dilution experiments performed on bone marrow mononuclear cells that had not been further fractionated and in an additional experiment performed on adherent and T cell–depleted bone marrow. From these experiments mean estimated precursor frequencies were calculated (Table 1). Both TNF and LT inhibited the response of cells to G-CSF such that the frequency of progenitor cells responding to G-CSF was reduced from one in 489 on day 7 and one in 1,015 on day 14 to less than one in 100,000 for both populations of cells. In contrast, neither TNF nor LT significantly altered the frequency of progenitors that responded to GM-CSF.

Colonies in microcultures stimulated by GM-CSF had the

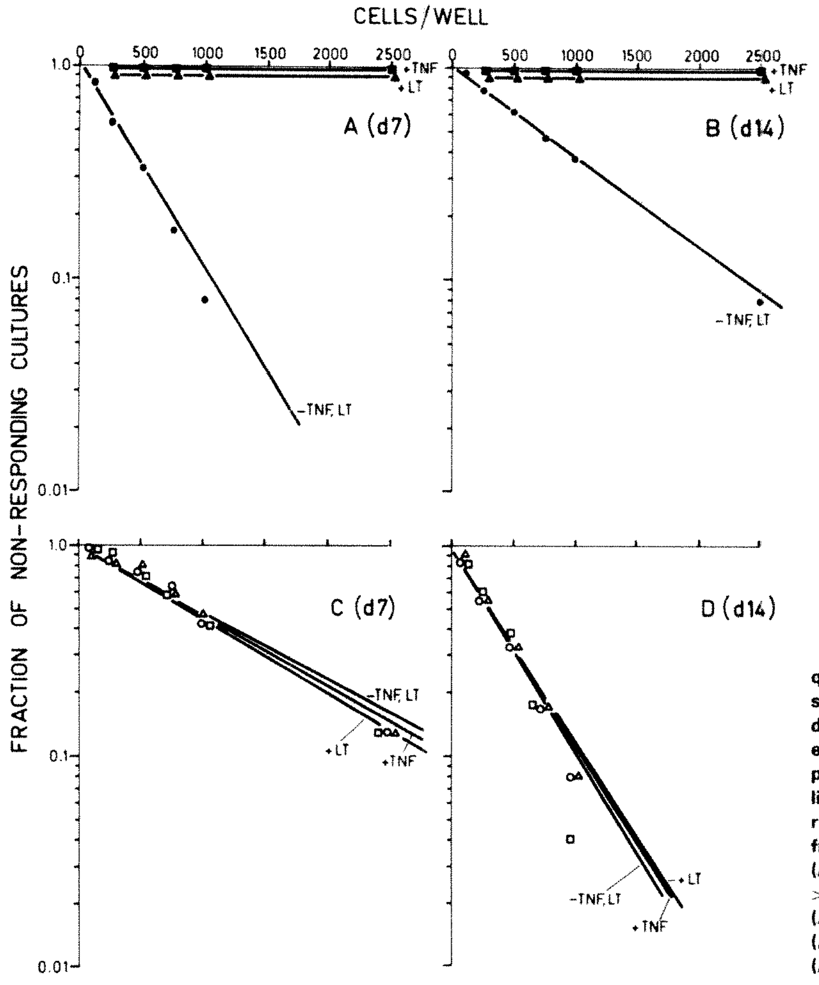

Fig 1. Effect of cytotoxins on progenitor frequencies for day 7 (A, C) and day 14 (B, D) colonies stimulated by G-CSF (A, B) or GM-CSF (C, D). The data shown are from one of four reproducible experiments. The lines were drawn such that the precursor frequency, calculated from maximum-likelihood determination, corresponded to a nonresponding culture fraction of 0.37. Reciprocals of frequency estimates were as follows: ●, d7, 453 (*P* = .74); d14, 1,020 (*P* = .99). ■▲, d7 and d14, >100,000. ○, d7, 1,361 (*P* = .93); d14, 452 (*P* = .93). □, d7, 1,355 (*P* = .90); d14, 460 (*P* = .74). △, d7, 1,313 (*P* = .99); d14, 461 (*P* = .77).

Table 1. Progenitor Frequencies in Human Bone Marrow

	Progenitor Cell Frequency	
Stimulus/Cytotoxin	Day 7	Day 14
G-CSF	1:489 ± 36	1:1,015 ± 41
G-CSF + TNF	<1:100,000	<1:100,000
G-CSF + LT		
GM-CSF	1:1,407 ± 124	1:574 ± 105
GM-CSF + TNF	1:1,375 ± 28	1:510 ± 71
GM-CSF + LT	1:1,437 ± 175	1:526 ± 91

Mean estimated progenitor frequencies for day 7 and day 14 colonies stimulated by G-CSF or GM-CSF in the absence or presence of cytotoxins. Means ± SD from three bone marrow samples are shown.

morphological appearances of pure granulocyte, pure macrophage, mixed granulocyte-macrophage, and eosinophil colonies. The relative proportions of these colony types were unaltered by either TNF or LT. The rare colony present in cultures stimulated by G-CSF in the presence of either TNF or LT was granulocytic in morphology.

DISCUSSION

The experiments described have given frequency estimates for late (day 7) and early (day 14) progenitor cells in adherent and T cell–depleted human bone marrow that are capable of proliferation in response to stimulation with either recombinant human G-CSF or recombinant human GM-CSF. These were one in 489 (day 7) and one in 1,015 (day 14) for G-CSF and one in 1,407 (day 7) and one in 574 (day 14) for GM-CSF. These frequencies are similar to those described for clonogenic cells in human bone marrow where light-density marrow cells were cultured with partially purified native G-CSF or GM-CSF.[14] The linearity of the plots and demonstration that the y-axis is intercepted at value 0 for both growth factors suggests that the effects of G-CSF and GM-CSF on progenitor cells are direct rather than indirect (mediated by accessory cells).

This study demonstrates the striking difference in sensitivity to cytotoxins of progenitor cells responsive to either G-CSF or GM-CSF. Clonogenic cells that proliferate after stimulation with GM-CSF are resistant to the suppressive effects of TNF (100 U/mL) and LT (100 U/mL), with no significant alteration in the frequency of responding marrow cells. A small suppressive effect in cultures stimulated with GM-CSF is noted when TNF or LT is added in doses of 1,000 U/mL.[12] In contrast, less than one in 100,000 cells are resistant to the effects of TNF or LT when cultures are stimulated with G-CSF.

A feature of the hematopoietic system is the overlapping functional properties of hematopoietic growth factors and apparent redundancy in the control of blood cell production. This is particularly so for G-CSF and GM-CSF, both of which stimulate the formation of granulocyte colonies and activate mature granulocyte function.[15,16] The mechanisms for the differential effect observed in the experiments described in this paper are therefore of considerable interest. One interpretation of the results is that the two growth factors, G-CSF and GM-CSF, act on largely separate populations of clonogenic cells that are different in their responses to the cytotoxins TNF and LT. Cells that proliferate in response to G-CSF are responsive to the effects of TNF and LT, whereas those cells capable of proliferation after stimulation with GM-CSF are relatively unresponsive to the cytotoxins. This mechanism, implicating separate progenitor populations, could result from differences in the expression of receptors for TNF and LT between the two cell pools. The observation that progenitors giving rise to both day 7 and day 14 colonies in response to G-CSF are both extremely sensitive to cytotoxins and, by contrast, that both sets of progenitors responsive to GM-CSF are resistant suggests that the effect is not due to cytotoxins acting on a more mature cell of the same granulocyte-macrophage progenitor pool that has increased responsiveness to G-CSF as compared with GM-CSF. Additional evidence supporting this is that cell clusters (aggregates of three to 39 cells) are less sensitive to the suppressive effects of the cytotoxins than are cell colonies.[12]

TNF and LT show considerable homology in amino acid sequence, bind to the same cell surface receptor, and share a number of biologic properties.[8-11] One possibility that might account for their difference in activity in the presence of G-CSF and GM-CSF may lie in effects on biochemical pathways associated with cell surface receptors for these two growth factors. There is only limited data on the structure of receptors for colony-stimulating factors and the intracellular pathways they might influence. The receptor for macrophage colony–stimulating factor possesses protein tyrosine kinase activity.[17] It is unclear whether G-CSF and GM-CSF receptors have an intrinsic protein kinase activity. Although the murine G-CSF receptor appears to have a molecular weight (mol wt) of approximately 150,000 daltons[18] and is large enough to include an intracellular protein kinase domain, the murine GM-CSF receptor has an estimated mol wt of only 50,000 to 70,000 daltons.[19,20] The intracellular domain of such a receptor may be too small to include a protein kinase component. It is possible that differences in the intracellular signaling systems activated after the binding of G-CSF and GM-CSF to their specific receptors may result in different interactions with TNF and LT. GM-CSF may be capable of antagonizing some or all of the effects of TNF and LT by acting at a postreceptor level. If the cytotoxins were to selectively influence the biochemical pathways associated with activation of receptors for G-CSF, it would not be possible to determine, from these experiments, whether G-CSF and GM-CSF were acting on the same, different, or overlapping populations of progenitor cells. There is experimental evidence from clone transfer studies that G-CSF and GM-CSF are capable of stimulating the same clone, though these experiments were conducted by using partially purified growth factors and require validation with fully purified molecules.[14] There are at least two potential mechanisms for a selective effect of TNF and LT on biochemical pathways activated after binding of G-CSF to its receptor. The effects could be either directly inhibitory or mediated by the induction of differentiation. The cytotoxins are capable of inducing cellular differentiation,[6,7] and G-CSF has been shown to be a powerful inducer of myeloid differentiation in vitro.[16,21] It is possible that TNF and LT, in synergy with G-CSF, have induced a rapid differentiation of progenitor cells at the expense of proliferation, with cell aggregates not reaching colony size or extinction of colonies before day 7.

Further investigation of the differential sensitivity of the granulocyte-macrophage system to the effect of cytotoxins may be helpful in further determining the roles of G-CSF and GM-CSF in regulating myeloid cell production.

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Proteoglycan Synthesis in Two Murine Bone Marrow Stromal Cell Lines

By Suzanne L. Kirby and Stuart A. Bentley

There is evidence indicating that stromal proteoglycans are an important functional component of the hematopoietic microenvironment. Proteoglycan synthesis was therefore investigated in the MS3-2A and D2XRII hematopoietic stromal cell lines. These lines differ in their capacity to support hematopoiesis in vitro, D2XRII supporting in vitro hematopoiesis, whereas MS3-2A does not. Cells were labeled with ^{35}S-sulfate as precursor, and 4 mol/L guanidine HCl extracts of cells and media were analyzed by ion-exchange chromatography, cesium chloride density gradient centrifugation, and molecular sieve chromatography. Proteoglycans were further examined by enzymatic and chemical digestions. MS3-2A cells produced at least three proteoglycan species. Two chondroitin/dermatan sulfate (CS/DS) proteoglycans, K_{av} = 0.40 and K_{av} = 0.68 on Sepharose CL-2B, were present primarily in the medium. The respective glycosaminoglycan molecular weight (mol wt) values were 38 kd and 40 kd. A heparan sulfate (HS) proteoglycan of K_{av} = 0.58 and glycosaminoglycan mol wt 36 kd was present primarily in the cell layer extract. D2XRII cells synthesized two HS proteoglycans. The larger (K_{av} = 0.45; glycosaminoglycan mol wt, 30 kd) was of low density on gradient centrifugation and more prominent in the cell layer extracts, whereas the smaller (K_{av} = 0.68; glycosaminoglycan mol wt, 38 kd) was dense and present mainly in the culture medium. A single CS/DS proteoglycan species of K_{av} 0.78 and average glycosaminoglycan of mol wt 18 kd was present in roughly equal amounts in the medium and in the cell layer. MS3-2A and D2XRII thus appear phenotypically distinct with respect to proteoglycan synthesis. These differences are discussed in relation to the microenvironmental function of bone marrow stromal elements.

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HEMATOPOIETIC STEM CELL function in vivo is dependent on close-range interaction with a complex cellular microenvironment.[1,2] The technique of long-term bone marrow culture[3] has provided a valuable in vitro model for study of the hematopoietic microenvironment at both the cellular and the molecular level. A variety of cellular phenotypes have been implicated as potential mediators of hematopoietic microenvironmental function,[4-9] and a variety of hematopoietic growth regulators have been demonstrated.[10-13]

There is evidence indicating that extracellular matrix components play a significant role in supporting hematopoiesis in long-term bone marrow culture. Proteoglycans are important components of extracellular matrices[14] and appear to have growth regulatory functions in a number of systems.[15] Proteoglycans are complex macromolecules consisting of a central protein core to which is attached a variable number of glycosaminoglycan side chains. Glycosaminoglycans are polysaccharides consisting of repeated disaccharide subunits that in turn consist of an amino sugar linked to a carboxylate, a sulfate ester, or both.[14] Chondroitin sulfate (CS) was found to be the major glycosaminoglycan in the medium and heparan sulfate (HS), the major glycosaminoglycan in the cell layer of murine long-term bone marrow cultures.[16] Perturbation of proteoglycan synthesis in murine long-term bone marrow culture by addition to the culture medium of *p*-nitrophenyl-β-D-xyloside[17] was shown to have profound effects on hematopoietic function[18] and lead to a fivefold increase in granulocyte/macrophage progenitors (CFU-G/M) and a tenfold increase in pluripotential hematopoietic stem cells (CFU-S). These changes were associated with increased levels of CS in the medium.[18]

Only a single major study has addressed the question of intact proteoglycan synthesis in long-term bone marrow culture.[19] Essentially similar patterns of proteoglycan synthesis were demonstrated in the cell layer and the medium, both of which were shown to contain at least three species of CS/dermatan sulfate (CS/DS) proteoglycans. CS and DS are closely related glycosaminoglycans, with glucuronic acid/*N*-acetyl galactosamine disaccharide subunits differing only in the degree of epimerization of glucuronic acid to iduronic acid. HS proteoglycan accounted for approximately 10% of the labeled material. Intact long-term bone marrow cultures contain a variety of cellular phenotypes, both stromal and hematopoietic, many of which have been shown to synthesize proteoglycans. It is not therefore possible to ascribe the synthesis of particular proteoglycan species to particular classes of cells.

An alternative approach for the investigation of stromal proteoglycan synthesis is the study of stromal cell lines derived from long-term bone marrow cultures. In the present study, we investigated two such stromal cell lines, namely, MS3-2A[20,21] and D2XRII,[22,23] the major properties of which are summarized in Table 1. These cell lines differ in one essential respect: D2XRII cells are microenvironmentally competent and support long-term in vitro hematopoiesis when cocultivated with hematopoietic progenitor cells.[23] MS3-2A cells are not microenvironmentally competent but appear to function as preosteoblasts responding to 1:3-dihydroxyvitamin D stimulation with greatly increased levels of alkaline phosphatase activity.[24]

MATERIALS AND METHODS

Materials. Guanidine HCl (practical grade), cesium chloride (optical grade), Sephadex G-50, diethyl aminoethyl (DEAE)-

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Table 1. Major Characteristics of the MS3-2A and D2XRII Stromal Cell Lines

Characteristic	MS3-2A	D2XRII
Interstitial collagens (types I, III)	++	+
Basement membrane collagen (type IV)	−	+
Fibronectin	+	+
Laminin	−	+
M-CSF	+	+
Multi-CSF	−	−
Hydrocortisone-induced adipogenesis	+	+
Preosteoblast properties	++	−
Microenvironmental competence	−	+

Sephacel, Sepharose (CL-2B, CL-4B, and CL-6B), 6-aminohexanoic acid, benzamidine HCl, *N*-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), CS (whale cartilage), chondroitinase ABC (*Proteus vulgaris*), and chondroitinase AC (*Arthrobacter aurescens*) were purchased from Sigma Chemical Co, St Louis; heparitinase (*Flavobacterium heparinum*) from ICN Immunobiologicals, Lisle, IL; sodium ^{35}S-sulfate from ICN Radiochemicals, Irvine, CA; Safety-Solve scintillation cocktail from Research Products International Corp, Mount Prospect, IL; fetal calf serum and sulfate-free RPMI 1640 medium from GIBCO, Grand Island, NY; L-glutamine, 1000× penicillin/streptomycin, HEPES, RPMI 1640, and medium 199 from the Tissue Culture Facility, University of North Carolina at Chapel Hill.

Cell culture techniques. The MS3-2A cell line was generously provided by Dr T.M. Dexter, Manchester, England. The D2XRII cell line was generously provided by Dr J.S. Greenberger, Worcester, MA. Cultures were maintained in 25-cm^2 tissue culture flasks at 34°C in a humidified atmosphere of 5% CO_2, 95% air. MS3-2A cells were cultured in medium 199, supplemented with 15% fetal calf serum, 1% 200 mmol/L L-glutamine, and penicillin/streptomycin 1000×. D2XRII cells were grown in RPMI 1640 containing 10% fetal calf serum, 1% 1 mol/L HEPES, 1% 200 mmol/L L-glutamine, and 1000× penicillin/streptomycin, under similar conditions. The cells reached confluence in four to five days and were subcultured by trypsinization, with a split ratio of 1:5.

Radioisotope labeling. Labeling was initiated as the cell cultures approached confluence by adding fresh medium containing either 50 μCi/mL or 200 μCi/mL sodium ^{35}S-sulfate as precursor. Sulfate-free RPMI 1640 was used in the labeling experiments.

Isolation of proteoglycans. After labeling, the cells were removed from the bottom of the flask with a cell scraper and the contents transferred to a centrifuge tube. The medium and cells were centrifuged at 1,000 g for 15 minutes at 5°C. The medium was decanted, and 0.53 g/mL solid guanidine HCl, 10 μL/mL 100× NEM, and 10 μL/mL 100× PMSF were added to make the solution 4 mmol/L in guanidine HCl, 10 mmol/L in NEM, and 1 mmol/L in PMSF. Cell pellets were extracted at room temperature with 4 mmol/L guanidine HCl, 0.1 mol/L 6-aminohexanoic acid, 10 mmol/L NEM, 1 mmol/L PMSF, 5 mmol/L benzamidine HCl, 50 mmol/L sodium EDTA, 50 mmol/L sodium acetate, and 0.5% Triton X-100, pH 5.8, 1 mL/25-cm^2 flask culture. The cell extracts and media were stored at −20°C for subsequent analysis. Unincorporated isotope was removed from the medium fractions and cell extracts by Sephadex G-50 chromatography (bed volume of 8 mL for each 1.5-mL sample). Columns were eluted with 8 mol/L urea, 0.05 mol/L sodium acetate, 0.15 mol/L sodium chloride, and 0.5% Triton X-100 for subsequent ion-exchange chromatography. Recoveries of macromolecular material were >85%, and separation from unincorporated material was complete.

Column chromatography. Sephadex G-50–excluded fractions in 8 mol/L urea, 0.15 mol/L sodium chloride, 0.5 mol/L sodium acetate, and 0.5% Triton X-100 were applied to DEAE-Sephacel columns (1.6 × 4 cm) equilibrated with the same 8 mol/L urea buffer. After sample application, the columns were washed with approximately 20 mL 8 mol/L urea buffer and then eluted with a linear NaCl gradient (0.15 to 1.0 mol/L) using a total volume of 100 mL. ^{35}S-labeled peaks were pooled and then concentrated on DEAE-Sephacel columns of 1.5-mL bed volume.[25] Recoveries of ^{35}S-labeled material were >80%.

Analytic columns of Sepharose CL-2B and CL-4B (1.6 × 100 cm) were prepared in and eluted with 4 mol/L guanidine HCl, 0.05 mol/L Tris, 0.05 mol/L sodium acetate, and 0.2% Triton X-100, pH 7.0, at a flow rate of 12 mL/h. Analytic columns of Sepharose CL-6B (1.6 × 100 cm) were prepared in and eluted with 0.1 mol/L sodium acetate and 0.1 mol/L Tris, pH 7.3 (flow rate, 12 mL/h). The void and total column volumes were marked with blue dextran and free ^{35}S-sulfate, respectively. Recoveries of labeled macromolecules were >80%.

Isopyknic cesium chloride centrifugation. Solid cesium chloride, 0.5 g/g, was added to medium and cellular proteoglycan fractions in 4 mol/L guanidine HCl buffer after ion-exchange chromatography and concentration to give an initial density of 1.40 g/mL. Dissociative density gradients were formed by using a Beckman 60 SW rotor at 37,000 rpm at 10°C for 48 hours in a Beckman L8-70 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Fifteen 0.25-mL fractions were collected after piercing the bottom of the tube. These fractions were pooled into four approximately equal fractions, designated D1 through D4 from bottom to top, for subsequent gel-filtration chromatography.

Chemical analyses. Alkaline borohydride treatment was performed in 0.05 mol/L sodium hydroxide and 1 mol/L sodium borohydride at 45°C for 24 hours, which resulted in β-elimination of glycosaminoglycan chains from the protein core.[26] Excess borohydride was destroyed by neutralization with glacial acetic acid. Nitrous acid treatment was performed at room temperature in 0.18 mol/L acetic acid with 0.24 mol/L sodium nitrite for 80 minutes.[27] The reaction was stopped by the addition of an equal volume of 2 mol/L ammonium sulfamate.

Enzymatic treatments. Samples were digested for four hours at 37°C with chondroitinase ABC (0.05 to 0.1 U/mg carrier) and chondroitinase AC (0.25U/mg carrier) in a 0.1 mol/L Tris, 0.1 mol/L sodium acetate buffer, pH 7.3.[28] Enzyme concentrations were adjusted for the content of CS carrier. Heparitinase digestion was carried out in a 0.1 mol/L Tris, 0.1 mol/L sodium acetate, and 0.005 mol/L calcium acetate buffer, pH 7.0, containing 0.5 mg/mL ovalbumin, 1 mmol/L PMSF, and 0.1 U/mL heparitinase.[29] Digestion was continued for three hours at 37°C and fresh enzyme added for one additional hour.

RESULTS

Kinetics of macromolecular incorporation of label. The accumulation of ^{35}S-labeled macromolecules in the media was linear for up to 96 hours but tended to plateau in the cell layer extracts (Fig 1). Preliminary gel-filtration chromatography indicated that proteoglycan synthesis was qualitatively stable for 96 hours, although there was progressive accumulation of smaller molecules, presumably proteoglycan degradation products, during the later stages of the observation period (data not shown). A 48-hour labeling period gave maximum incorporation of isotope with acceptable levels of proteoglycan degradation and was therefore considered optimal for subsequent experiments. At the 48-hour time point, the ratio of macromolecular ^{35}S activity in the media to that in the cell layer extracts was approximately 2:1.

Analysis of culture media proteoglycans. DEAE-

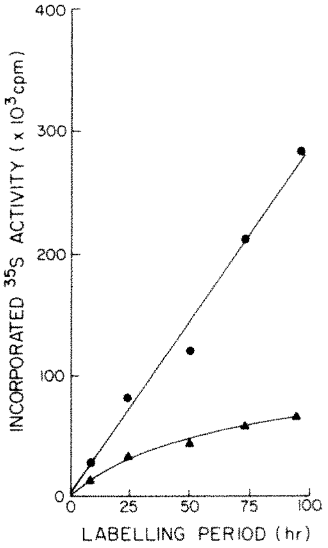

Fig 1. Accumulation of ^{35}S-labeled macromolecules in the medium (●) and cell layer (▲) of MS3-2A cultures. Virtually identical results were obtained in D2XRII cultures.

Table 2. Analysis of CsCl Density Gradient Fractions From MS3-2A and D2XRII Culture Medium

Fraction	Density (g/L)	Percent of Total Gradient Activity	CS/DS Proteoglycan Percent of Fraction	CS/DS Proteoglycan K_{av}	HS Proteoglycan Fraction (%)	HS Proteoglycan K_{av}
MS3-2A D1	1.59	25	46	0.36, 0.70	47	0.62
MS3-2A D2	1.48	34	64	0.42, 0.69	28	0.60
MS3-2A D3	1.39	28	73	0.38, 0.67	37	0.62
MS3-2A D4	1.33	13	62	0.60	43	0.69
D2XRII D1	1.58	22	20	0.85	80	0.66
D2XRII D2	1.48	33	40	0.82	60	0.69
D2XRII D3	1.39	24	65	0.78	30	0.62
D2XRII D4/I	1.31	9	42	0.64	66	0.55
D2XRII D4/II	1.31	12	70	0.80	20	*

CS/DS proteoglycans were quantitated as chondroitinase ABC-sensitive activity and HS proteoglycans, as heparitinase-sensitive activity.

*Scanty residual material with no clearly defined peak.

Sephacel chromatography of macromolecular material from both MS3-2A and D2XRII culture media revealed single, broad ^{35}S-labeled peaks eluting between 0.40 and 0.65 mol/L NaCl (data not shown). Pooled material from these peaks was further analyzed by CsCl density gradient centrifugation. Sepharose CL-2B chromatograms of the gradient fractions from the MS3-2A culture medium are illustrated in Fig 2A. All the gradient fractions contained proteoglycans of K_{av} 0.65 to 0.70 in substantial quantities, with a tendency to decreasing hydrodynamic size with decreasing density. The D1 to D3 fractions also contained proteoglycans of K_{av} 0.35 to 0.45, whereas the D4 fractions contained an incompletely resolved peak of intermediate hydrodynamic size. Sepharose CL-2B chromatograms of the gradient fractions from the D2XRII culture medium are illustrated in Fig 2B. All D2XRII medium fractions contained a major labeled peak in the range 0.65 to 0.75 K_{av}, whereas the medium D4 fraction also contained a clearly defined second peak of K_{av} 0.55.

The percentages of ^{35}S-labeled material in each culture medium fraction sensitive to chondroitinase ABC and heparitinase digestion and eluting at Vt (total column volume) on subsequent Sepharose CL-2B chromatography are shown in Table 2 together with K_{av} values for the resistant material. Digestion data indicate that all MS3-2A medium fractions contained a polydisperse CS/DS proteoglycan of K_{av}, 0.68 that accounted for 45% of the total radioactivity recovered from the gradient. The intermediate-density fractions (mainly D2 and D3) also contained a larger CS/DS proteoglycan (K_{av}, 0.35 to 0.45) that accounted for 20% of the recovered activity. In addition, all fractions contained a polydisperse HS proteoglycan (K_{av}, 0.61) that accounted for 35% of the activity recovered from the gradient. Digestion data for D2XRII culture medium indicate that the major peaks in all fractions (K_{av}, 0.65 to 0.75) contained varying

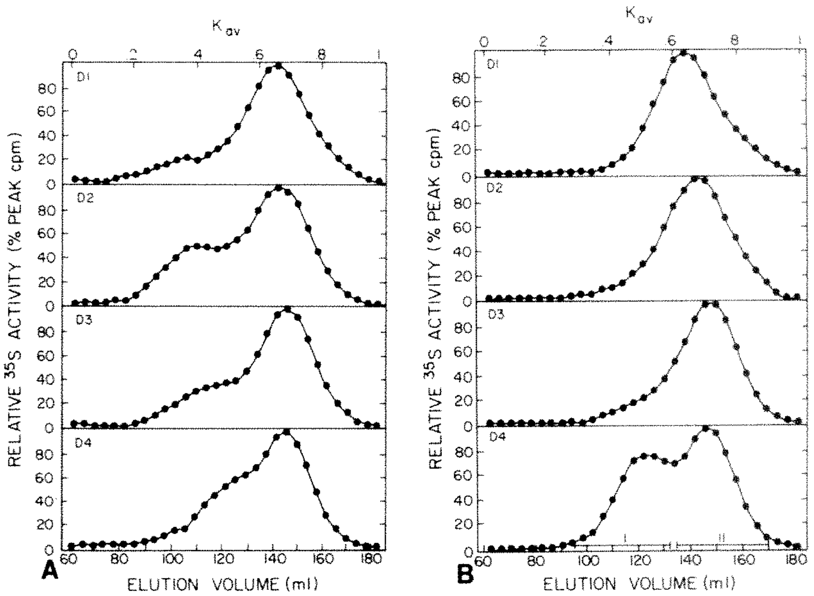

Fig 2. Sepharose CL-2B chromatograms of CsCl gradient fractions from MS3-2A and D2XRII culture media. All MS3-2A gradient fractions (A) contained proteoglycans of K_{av} 0.65 to 0.70 in substantial quantities. The D1 to D3 fractions also contained proteoglycans of K_{av} 0.35 to 0.45, whereas the D4 fraction contained an incompletely resolved peak of intermediate hydrodynamic size. All D2XRII medium fractions (B) contained a major labeled peak in the range 0.65 to 0.75 K_{av}. In addition, the D4 fraction contained a clearly defined second peak of K_{av} 0.55 (B).

proportions of a dense HS proteoglycan of K_{av} 0.67 and a somewhat less dense polydisperse CS/DS proteoglycan (K_{av} 0.78). These proteoglycans accounted, respectively, for 45% and 49% of the total activity recovered from the gradient. Analysis of the D4 gradient fraction also revealed the presence of a larger, low-density HS proteoglycan (K_{av}, 0.55) that accounted for approximately 6% of the total recovered radioactivity.

Proteoglycans from MS3-2A and D2XRII culture medium were digested with chondroitinase ABC or heparitinase and the resistant material separated on Sepharose CL-2B. The resulting peaks (see Fig 2) were pooled, concentrated by ultrafiltration, and subjected to alkaline borohydride treatment for subsequent glycosaminoglycan analysis. The Sepharose CL-6B elution profile for MS3-2A culture medium HS glycosaminoglycan showed a single peak of K_{av} 0.37 that corresponded to a molecular weight (mol wt) of 36 kd. The elution profile for D2XRII medium HS glycosaminoglycan showed a single peak of K_{av} 0.36 that corresponded to a mol wt of 38 kd. Both peaks were fully susceptible to nitrous acid digestion, which confirmed that the chondroitinase ABC–resistant material in both original samples was HS proteoglycan. The larger, low-density HS proteoglycan was purified separately from the D2XRII medium D4 fraction and subjected to alkaline borohydride treatment. A subsequent Sepharose CL-6B chromatogram showed a single included peak of K_{av} 0.35 that corresponded to a glycosaminoglycan mol wt of 40 kd. The elution profiles of glycosaminoglycans isolated from the two MS3-2A medium CS/DS proteoglycans were virtually identical to each other and showed single included peaks of K_{av} 0.36 that corresponded to a glycosaminoglycan mol wt of 38 kd. Glycosaminoglycan chains from the D2XRII medium CS/DS proteoglycan were considerably smaller and gave a single included peak of K_{av} 0.47 that corresponded to a mol wt of 22 kd.

Analysis of cell layer extract proteoglycans. DEAE-Sephacel elution profiles of macromolecular material obtained from MS3-2A and D2XRII cell layer extracts showed single, broad ^{35}S-labeled peaks eluting between 0.40 mol/L and 0.65 mol/L NaCl (data not shown). Pooled material from these peaks was further analyzed by CsCl density gradient centrifugation. Sepharose CL-2B chromatograms of the gradient fractions from MS3-2A cell layer extract are illustrated in Fig 3A. Chromatograms of all fractions showed peaks of K_{av} 0.55 to 0.60. Fractions D1 to D3 contained a small amount of a proteoglycan species of similar hydrodynamic size to that seen in the equivalent medium fractions (K_{av}, 0.35 to 0.45). Fraction D1 also contained an incompletely resolved peak of K_{av} 0.73. Sepharose CL-2B chromatograms of the CsCl gradient fractions from D2XRII cell layer extract are illustrated in Fig 3B. The D1 to D3 fractions each showed two major peaks of K_{av} 0.55 to 0.60 and K_{av} 0.78, respectively. The K_{av} 0.78 peak predominated in fraction D1, whereas the K_{av} 0.55 to 0.60 peak predominated in fraction D3. The D4 fraction showed peaks of K_{av} 0.45 and 0.62.

The percentages of ^{35}S-labeled material in each cell layer extract fraction sensitive to chondroitinase ABC and heparitinase digestion and eluting at Vt on subsequent Sepharose CL-2B chromatography are shown in Table 3 together with K_{av} values for the resistant material. Digestion data for the MS3-2A cell layer extract indicate that all fractions contained a HS proteoglycan of K_{av} 0.55 to 0.60, although the majority of this proteoglycan was present in the low-density fractions (D3 and D4). HS proteoglycan accounted for approximately 45% of the total radioactivity recovered from the gradient. The K_{av} 0.35 to 0.45 material in gradient fractions D1 to D3 was a large CS/DS proteoglycan similar to that found in MS3-2A culture medium, and this accounted for approximately 10% of the total recovered radioactivity. Fractions D2 to D4 contained a CS/DS proteoglycan of K_{av} 0.65 to 0.70 that accounted for about 35% of total recovered radioactivity. The K_{av} 0.73 peak in gradient fraction D1 was fully sensitive to chondroitinase ABC digestion and accounted for 10% of the total recovered radioactivity. On Sepharose CL-6B chromatography before and after alkaline borohydride treatment, the observed K_{av} values were 0.36 and 0.44, respectively. This suggests that the material in

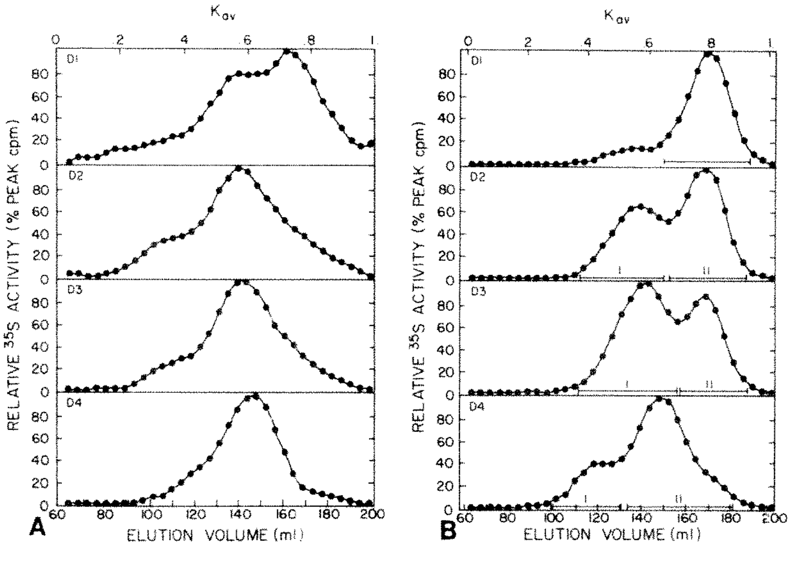

Fig 3. Sepharose CL-2B chromatograms of CsCl gradient fractions from MS3-2A and D2XRII cell layer extracts. MS3-2A cell layer (A) fractions D1 to D3 contained proteoglycans in the range of K_{av} 0.35 to 0.45. All fractions contained peaks of K_{av} 0.55 to 0.60. Fraction D1 also contained an incompletely resolved peak of K_{av} 0.73. The D2XRII cell layer (B) D1 to D3 fractions each showed two major peaks of K_{av} 0.55 to 0.60 and K_{av} 0.78, respectively, the K_{av} 0.78 peak predominating in fraction D1 and the K_{av} 0.55 to 0.60 peak, in fraction D3. The D4 fraction showed peaks of K_{av} 0.45 and 0.62.

Table 3. Analysis of CsCl Density Gradient Fractions From MS3-2A and D2XRII Cell Layer Extracts

Fraction	Density (g/L)	Percent of Total Gradient Activity	CS/DS Proteoglycan		HS Proteoglycan	
			Percent of Fraction	K_{av}	Fraction (%)	K_{av}
MS3-2A D1	1.60	30	76	0.35, 0.75	35	0.56
MS3-2A D2	1.49	32	68	0.36, 0.70	51	0.52
MS3-2A D3	1.40	21	43	0.35, 0.69	67	0.61
MS3-2A D4	1.35	17	40	0.65	70	0.55
D2XRII D1/I	1.59	5	33	*	75	0.55
D2XRII D1/II	1.59	25	85	0.82	20	*
D2XRII D2/I	1.49	12	13	0.66	85	0.58
D2XRII D2/II	1.49	20	85	0.81	20	0.85
D2XRII D3/I	1.42	13	19	0.69	79	0.59
D2XRII D3/II	1.42	8	80	0.82	35	0.82
D2XRII D4/I	1.33	5	20	*	85	0.55
D2XRII D4/II	1.33	12	60	0.73	35	0.67

CS/DS proteoglycans were quantitated as chondroitinase ABC-sensitive activity and HS proteoglycans, as heparitinase-sensitive activity.

*Scanty residual material with no clearly defined peak.

Table 4. Summary of Major Characteristics of Proteoglycans Identified in MS3-2A and D2XRII Culture Media

Designation	Source (Medium)	Percent of Source PG	K_{av} CL-2B	Density	GAG	GAG mol wt (kd)
MS3-M-DS1	MS3-2A	19	0.35-0.40	Int	DS	38
MS3-M-DS2	MS3-2A	45	0.68	Poly	DS	40
D2X-M-DS	D2XRII	49	0.78	Int	DS	22
MS3-M-HS	MS3-2A	36	0.61	Poly	HS	36
D2X-M-HS1	D2XRII	45	0.67	High	HS	38
D2X-M-HS2	D2XRII	6	0.55	Low	HS	40

Abbreviations: PG, proteoglycan; GAG, glycosaminoglycan; Int, intermediate; Poly, polydisperse.

question was a small CS/DS proteoglycan rather than a free glycosaminoglycan and that the corresponding glycosaminoglycan mol wt was 26 kd.

Digestion data for the D2XRII cell layer extract indicate the presence of an intermediate- to low-density HS proteoglycan of K_{av} 0.58 that accounted for 34% of the ^{35}S activity recovered from the gradient. The D4 fraction also contained a low-density HS proteoglycan of K_{av} 0.45 that appeared distinct from the K_{av} 0.58 material. This accounted for approximately 8% of the recovered activity. An intermediate- to high-density CS/DS proteoglycan of K_{av} 0.78 accounted for approximately 58% of the total activity recovered from the gradient.

Cell layer proteoglycans were digested with chondroitinase ABC or heparitinase and the resistant material separated on Sepharose 2B-CL. The resulting peaks (see Fig 3) were pooled, concentrated by ultrafiltration, and subjected to alkaline borohydride treatment for subsequent glycosaminoglycan analysis. The Sepharose CL-6B elution profile for MS3-2A cell layer HS glycosaminoglycan showed a single peak of K_{av} 0.37 that corresponded to a mol wt of 36 kd. This material was fully susceptible to subsequent nitrous acid digestion. The MS3-2A cell layer HS glycosaminoglycan was thus equal in size to the MS3-2A medium HS glycosaminoglycan, although the intact cell layer proteoglycan was somewhat larger and more buoyant. The elution profiles of glycosaminoglycans isolated from the two MS3-2A cell layer CS/DS proteoglycans were virtually identical to each other and showed single included peaks of K_{av} 0.36 that corresponded to a glycosaminoglycan mol wt of 38 kd. These results were in turn identical to those obtained for the corresponding proteoglycans isolated from MS3-2A medium.

The elution profile for D2XRII cell layer HS glycosaminoglycan showed a single peak of K_{av} 0.41 that corresponded to a mol wt of 30 kd. This material was fully susceptible to subsequent nitrous acid digestion. The larger, low-density HS proteoglycan was purified from the D2XRII cell layer D4 fraction and subjected to alkaline borohydride treatment. A subsequent Sepharose CL-6B chromatogram also showed a single included peak of K_{av} 0.41 that corresponded to a glycosaminoglycan mol wt of 30 kd. The elution profile of glycosaminoglycan isolated from the D2XRII cell layer CS/DS proteoglycan showed a single included peak of K_{av} 0.52, that corresponded to a glycosaminoglycan mol wt of 15 kd.

DISCUSSION

The major characteristics of the proteoglycans identified in MS3-2A and D2XRII culture media are summarized in Table 4. MS3-2A secreted two clearly identifiable CS/DS proteoglycans differing in size and buoyant density. In contrast, D2XRII cells produced only a single CS/DS proteoglycan that was smaller and more dense than the corresponding MS3-2A proteoglycan (MS3-M-DS2), with smaller glycosaminoglycan side chains. D2XRII cells secreted relatively more HS proteoglycan into the culture medium than did MS3-2A cells. In further contrast, the D2XRII medium HS proteoglycans were readily resolved into a small, high-density species (D2X-M-HS1) and a larger, low-density species (D2X-M-HS2), whereas only a single, polydisperse HS proteoglycan was identifiable in MS3-2A culture medium.

The major characteristics of the proteoglycans identified in MS3-2A and D2XRII cell layer extracts are summarized in Table 5. The D2XRII cell layer contained only a single CS/DS proteoglycan, closely resembling that identified in the culture medium. In contrast, three CS/DS proteoglycans were identified in the MS3-2A cell layer. Two of these (MS3-C-DS1 and MS3-C-DS2) were similar to correspond-

Table 5. Summary of Major Characteristics of Proteoglycans Identified in MS3-2A and D2XRII Cell Layer Extracts

Designation	Source (Cell)	Percent of Source PG	K_{av} CL-2B	Density	GAG	GAG mol wt (kd)
MS3-C-DS1	MS2-2A	10	0.35-0.40	Int	DS	38
MS3-C-DS2	MS3-2A	25	0.65-0.70	Poly	DS	40
MS3-C-DS3	MS3-2A	10	0.73	High	DS	26
D2X-C-DS	D2XRII	58	0.78	High	DS	15
MS3-C-HS	MS3-2A	55	0.55-0.60	Low	HS	36
D2X-C-HS1	D2XRII	34	0.58	Int	HS	30
D2X-C-HS2	D2XRII	8	0.45	Low	HS	30

ing proteoglycans in the culture medium (MS3-M-DS1 and MS3-M-DS2), whereas a third CS/DS proteoglycan (MS3-C-DS3) more closely resembled the D2XRII cell layer CS/DS proteoglycan. Both cell layers contained low-density HS proteoglycans. The D2XRII cell layer HS proteoglycans were further resolved into intermediate- and low-density fractions (D2X-C-HS1 and D2X-C-HS2). It is uncertain whether these represent distinct proteoglycan species or polydispersity of a single species.

The present studies have demonstrated substantial differences between the patterns of proteoglycan synthesis in MS3-2A and D2XRII cells. The proteoglycan synthesis pattern of MS3-2A cells had much in common with that observed in a variety of fibroblast cell lines. Vogel and Peterson,[30] studying proteoglycan synthesis in IMR-90 human embryonic lung fibroblasts, identified two CS/DS proteoglycans in the medium of K_{av} 0.29 and K_{av} 0.68 on Sepharose CL-2B. The major proteoglycan species found in the cell layer was a low-density HS proteoglycan of K_{av} 0.40 on Sepharose CL-2B. Carlstedt et al[31,32] found a large, glucuronic acid–rich DS proteoglycan and a smaller, iduronic acid–rich DS proteoglycan to be the major species in the culture medium from embryonic skin fibroblasts. The cells contained mainly HS proteoglycan, a DS proteoglycan similar to the large DS proteoglycan from the medium and free DS chains. The proteoglycan synthesis pattern of D2XRII cells more closely resembled that observed in endothelial cells. Bovine aortic endothelial cells have been shown to synthesize a large buoyant HS proteoglycan, a smaller dense HS proteoglycan of K_{av} 0.43 on Sepharose CL-4B, and a CS/DS proteoglycan of K_{av} 0.45.[33]

It is tempting to associate differences in proteoglycan synthesis with functional differences between these cell lines. MS3-2A cells appear to represent a fibroblast-like phenotype with the potential to develop into osteoblasts.[24] The osteoblastic potential of so-called bone marrow fibroblasts is well documented,[34] and it has further been demonstrated that these cells do not provide a microenvironment supportive of hematopoiesis in long-term bone marrow culture.[5] Because the proteoglycan synthesis pattern of D2XRII cells has more in common with the endothelial cell phenotype, the present findings are in accordance with the current consensus that the hematopoietic microenvironment in long-term bone marrow culture is provided by an endothelial-like cell.

Hematopoietically active human long-term bone marrow cultures were shown to produce three species of CS/DS proteoglycan on the basis of hydrodynamic size, with similar heterogeneity in the medium and the cell layer.[19] A large, glucuronic acid–rich species eluted in the void volume of a Sepharose CL-4B column. The remaining CS/DS proteoglycan appeared to consist of an intermediate-sized glucuronic acid–rich fraction excluded from a Sepharose CL-6B column and a smaller species, K_{av} 0.39 on Sepharose CL-6B, richer in iduronic acid. HS proteoglycan accounted for only 10% of the labeled material in this culture system. It was suggested that this pattern of proteoglycan synthesis resembled that of smooth muscle cells, which have previously been proposed as potential contributors to hematopoietic microenvironmental function.[9] Smooth muscle cells have been shown to produce two CS/DS proteoglycans, including a large, glucuronic acid–rich species of K_{av} 0.30 on Sepharose CL-2B and a smaller, iduronic acid–rich species of K_{av} 0.69, with only small quantities of HS proteoglycan.[35] Thus smooth muscle cells, in terms of proteoglycan synthesis, more closely resemble MS3-2A cells, which are incompetent microenvironmentally, than D2XRII cells. Although the presence of smooth musclelike cells in long-term bone marrow cultures is certainly a possibility, it is probable that the observed proteoglycan heterogeneity reflected the presence of multiple stromal and hematopoietic phenotypes. Synthesis of proteoglycan, especially CS proteoglycan, has been demonstrated in lymphocytes,[36,37] monocytes,[37,38] neutrophils,[39,40] eosinophils,[41] and basophils.[42] Proteoglycans derived from hematopoietic elements may thus represent a substantial proportion of total proteoglycan synthesis in hematopoietically active long-term bone marrow cultures.

There is compelling evidence that proteoglycans contribute significantly to the functional hematopoietic microenvironment.[18] To evaluate this contribution, it will be necessary to identify and characterize the specific proteoglycans involved. Proteoglycans are extremely complex structures and are therefore polydisperse. There is thus considerable overlap between different species with respect to chemical and physical properties, and resolution of individual proteoglycans is difficult, even in pure culture systems. In a heterogeneous system such as long-term bone marrow culture, such resolution is virtually impossible. The study of microenvironmentally competent stromal cell lines offers a potentially valuable alternative.

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Hematopoetic Precursors Respond to a Unique B Lymphocyte–Derived Factor In Vivo

By Eero Niskanen, Janet Gorman, and Peter C. Isakson

In this study we detected a factor that stimulates the proliferation of bone marrow–derived hematopoietic precursors in diffusion chambers implanted in mice. This factor, called diffusible colony-stimulating factor (D-CSF), was found in medium conditioned in the presence of spleen and peripheral blood cells from mice with B cell leukemia (BCL$_1$). After the administration of D-CSF, the number of colonies formed in the plasma clot inside the chamber (CFU-DG) was increased, as were the number of hematopoietic precursors (CFU-MIX, CFU-S, CFU-C, and BFU-E) as judged by a subculture of diffusion chamber contents. Depletion of macrophages and T cells from the spleen cell suspension did not decrease the production of D-CSF, thereby indicating that it was derived from B cells. Neoplastic BCL$_1$ cells appear to be the source because D-CSF could not be detected in medium conditioned with normal B cells. BCL$_1$-conditioned medium (CM) did not enhance CFU-MIX, BFU-E, and CFU-C colony formation in vitro, which suggested that D-CSF is different from multi-CSF, EPA, or CSF. The addition of BCL$_1$ CM to multi-CSF–, erythroid potentiating activity (EPA), and CSF (EL-4CM)-containing cultures had no effect on CFU-MIX, BFU-E, and CFU-C colony formation, thus indicating the absence of a synergistic or inhibitory activity. On the other hand, EL-4 CM, which stimulates CFU-MIX, BFU-E, and CFU-C in vitro, had no effect on CFU-DG in vivo. Biochemical characterization of BCL$_1$ CM revealed that D-CSF is relatively heat stable and loses its bioactivity with protease treatments. It binds to lentil-lectin, according to gel-filtration chromatography has a relative molecular weight of approximately 43,000, and on reverse-phase high-performance liquid chromatography elutes with acetonitrile. These data also indicate that transformed B cells may serve as a source for hematopoietic regulators that act on hematopoietic precursors in vivo.

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THE FORMATION OF COLONIES in semisolid cultures in vitro has been established as a standard probe to detect hematopoietic activities. Based on morphological assessment, cytokines have been identified that induce single cells to give rise to progeny consisting of erythroid, granulocytic, monocytic, megakaryocytic, or mixed populations of cells.[1-8] Whether hematopoietic cell proliferation in vivo is modulated by the same substances has been difficult to determine, except for the role of erythropoietin on red cell production. The existence of other physiological hematopoietic regulators active in vivo has been implied by enhanced granulocytic colony formation from bone marrow in diffusion chambers implanted in mice that are rendered cytopenic with cytotoxic agents or total body irradiation.[9,10] The murine bone marrow cells (CFU-DG) that give rise to these colonies can also give rise to multipotential precursor cells (12-day CFU-S),[11] which suggests that the diffusion chamber system detects activities early in hematopoietic differentiation.

Previously, we have discovered that two neoplastic human T cell lines (Mo and HUT 102) elaborate an activity that we call diffusible colony-stimulating factor (D-CSF), which enhances granulocytic colony formation from human marrow in diffusion chambers in mice. This activity can be separated from those activities that stimulate multipotential (CFU-MIX) and unipotential (BFU-E, CFU-C) precursors in vitro.[12,13] The unresponsiveness of murine hematopoietic cells to the human factor has limited further in vivo characterization of D-CSF and indicates species specificity. This obstacle is now removed by identification of murine B cell line leukemia, BCL$_1$, as a source of D-CSF.

MATERIALS AND METHODS

Cell preparation. The BCL$_1$ cell line is monoclonal and bears abundant surface IgM$_1$.[14,15] It is maintained by intravenous passage of 2×10^6 peripheral blood cells obtained from BCL$_1$-bearing BALB/c mice (Cumberland View Farms, Clinton, TN).

BCL$_1$ conditioned medium. The BCL$_1$ spleen cells and peripheral blood cells were obtained from BALB/c mice inoculated with BCL$_1$ cells 4 to 6 weeks earlier. After separation on a Ficoll-Hypaque gradient, macrophages were removed by adherence (30 minutes, four times) to glass and T cells by anti-Thy 1.2 antibody (Immunology Associates, Westchester, PA) plus baby rabbit complement. After these treatments, the preparations contained less than 0.3% macrophages and T cells as judged by nonspecific esterase stain and fluorescence microscopy, respectively. Purity of the BCL$_1$ cells was also assessed by fluorescence microscopy after staining with rhodamine-conjugated polyvalent Ig goat antimouse immunoglobulins. Pure populations of normal B cells were obtained similarly from the spleens of normal BALB/c mice. Then the cells were incubated in 75-cm^2 plastic flasks (Corning Glass Works, Corning, NY) at 37°C and 10% CO_2 in RPMI 1640 medium with or without 10% fetal calf serum (FCS) for six days at 2×10^6 cells/mL. In the absence of serum the cultures were supplemented with 1% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis) and 10 mM HEPES buffer (GIBCO, Grand Island, NY). During the culture period, cell viability remained high (>90%), and there was no net increase in cell number, which indicated a relatively quiescent cell population. Reverse transcriptase assay of BCL$_1$ conditioned medium (CM) did not demonstrate any increased activity, which suggested that viruses or virus-derived particles were not responsible for the hematopoietic effects. Endotoxin was removed from selected preparations by using the limulus amebocyte lysate absorption technique.[16]

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EL-4 CM. EL-4 CM was obtained by culture of EL-4 cells (10^6/mL) in 2% FCS and RPMI 1640 with 10 nmol/L phorbol myristate acetate for two to three days. The CM was concentrated tenfold by a Millipore CS-30 apparatus and then dialyzed extensively against phosphate-buffered saline (PBS). This CM contained high levels of interleukin 3 (IL-3), IL-2, GM-CSF, and B cell stimulating factor (BSF-1).[17]

CFU-DG assay. The number of CFU-DG was determined by the plasma clot diffusion chamber technique.[18] Diffusion chambers were prepared by gluing Millipore filters (Millipore Corp, Bedford, MA; pore size, 0.22 uμ) onto both sides of a plastic ring. Each chamber was filled with 0.1 mL murine bone marrow cell suspension (concentration 1×10^6 cells/mL). After sealing, two chambers were implanted into the peritoneal cavity of each mouse. Preparations to be assessed were given either intraperitoneally (0.5 mL/d) or by osmotic minipumps (Alza Corp, Palo Alto, CA), which were implanted in the peritoneal cavity. At the end of the culture, six to eight chambers per group were removed from the hosts. The clot that had formed inside the chamber was fixed in 5% glutaraldehyde and stained with benzidine-hematoxylin, dried, and made transparent by dipping in immersion oil. On microscopic examination, cell aggregates containing more than 19 granulocytic cells or more than seven erythroid cells were counted as colonies. Based on a dilution curve, the amount of protein giving rise to one colony over control was considered 1 unit.

CFU-C assay. The double-layer agar technique was used to assay CFU-C.[19] Bone marrow cells (1×10^5/plate) were placed in the upper layer, and the agent to be assayed for stimulating activity was placed in the lower layer. In assays for inhibitory activity, bone marrow cells and compounds to be assayed were placed in the same single layer. Triplicate cultures were incubated in 5.0% CO_2, and the colonies were counted after seven days.

BFU-E assay. Cells were cultured in Petri dishes, by using the methylcellulose technique of Iscove et al.[20] Human urinary erythropoietin was obtained from Toyobo, Inc (Osaka, Japan). Duplicate plates were incubated at 37°C in a humidified atmosphere of 7% CO_2 in air. On day 8, aggregates of 40 cells or more were counted as BFU-E.

CFU-MIX assay. A technique first described by Fauser and Messner[21] and modified by Ash et al[22] was used to assay CFU-MIX. Cells were cultured in 35-mm Petri dishes with 5% CM from EL-4 cell line, 1 unit of human erythropoietin (Toyobo), 30% FCS, Iscove's 10^{-5} modified Dulbecco's medium, 5×10^{-5} mol/L 2-mercaptoethanol (Sigma Chemical Co, St Louis), and 0.9% methylcellulose as a viscous support. After 14 days of incubation at 37°C in a humid atmosphere containing 5% CO_2, cultures were examined for the presence of mixed colonies. Mixed colonies were identified by their composition of translucent and hemoglobinized cells. Heterogenous morphology was verified on slides prepared from individual colonies and stained with Wright's stain.

CFU-S assay. CFU-S were assessed by the method described by Till and McCulloch.[23] Host mice were pretreated with 850-R total body irradiation. Each mouse received 0.25 mL of cell suspension (concentration, 3.2×10^5 cells/mL). They were killed 12 days later, and their spleens were removed and fixed in Bouin's solution for colony count.

Culture of hematopoietic cells in diffusion chambers in mice. The diffusion chamber technique used in this study has been described in detail elsewhere.[18] Diffusion chambers were filled with 1×10^5 normal murine bone marrow cells and implanted intraperitoneally in mice. The hosts were injected intraperitoneally daily with 0.5 mL of media to be tested. After four days of implantation, six chambers per group were removed and placed in McCoy's medium containing 0.5% pronase (Calbiochem, La Jolla, CA) and 5% Ficoll (Sigma) to dissolve the clot formed inside the chambers. After agitation for 80 minutes at room temperature, the chambers were opened sterilely, and the contents were removed for determination of CFU-S, CFU-MIX, BFU-E, and CFU-C concentrations per chamber.

Enzyme treatments. BCL$_1$ CM was treated with trypsin (1.8 U/mL in 0.1 mol/L Tris-HCL for 20 minutes, Sigma), chymotrypsin (0.13 U/mL in 0.1 mol/L Tris-HCL for 20 minutes, Sigma), pronase (0.14 U/mL in PBS for 20 minutes, Calbiochem), or neuraminidase (8 U/mL in 1 mol/L sodium acetate for 90 minutes, Sigma).

Purification of D-CSF. BCL$_1$ CM was concentrated tenfold by a mini-ultrasart ultrafiltration module (Sartorius, Göttingen, West Germany) and dialyzed against PBS. This material was applied to a column (2.5 × 6 cm) of lentil-lectin Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) that had been equilibrated with PBS. Then 10 vols of buffer was used to wash the column, and 35 mL of 0.2 mol/L methyl-D-mannoside in PBS was used to elute D-CSF. The resulting preparation was again concentrated tenfold, applied to an Ultrogel AcA44 column (1.5 × 90 cm; LKB, Gaithersberg, MD), and equilibrated with PBS as the running buffer. The column was calibrated by using ovalbumin (43 kd) and chymotrypsinogen (25 kd). The flow rate was 6 mL/h, and 3-mL fractions were collected. Fractions with the highest D-CSF activity were combined and applied directly to the high-performance liquid chromatography (HPLC) column without additional manipulation. Reverse-phase HPLC was performed on a Beckman apparatus (Beckman Instruments, Inc, Fullerton, CA) by using a Brownlee C-8 column (4.5 × 100 mm; Brownlee, Santa Clara, CA) with a starting solvent of 10% acetonitrile and 0.1% trifluoroacetic acid in water. The column was eluted with increasing concentrations of acetonitrile in 0.1% trifluoracetic acid by using the following gradient: 10% to 30% acetonitrile over 20 minutes and then 30% to 60% over 60 minutes. The flow rate was 1.5 mL/min. UV absorbance was monitored at 214 nm, and protein peaks were collected by a FRAC-100 fraction collector (Pharmacia), evaporated to dryness, and resuspended in a small volume of PBS.

Protein determination. The protein concentration was measured by the Bradford method.[24]

RESULTS

Effect of BCL$_1$ on granulopoiesis in vivo. BALB/c mice inoculated with BCL$_1$ demonstrate, in accordance with an earlier study (Muirhead, personal communication), an increase in the granulocyte count (Fig 1); the hematocrit value and platelet count remained unchanged up to 5 weeks. To determine whether the granulocytosis might be caused by an activity stimulating colony formation inside diffusion chambers, we implanted diffusion chambers containing 1×10^5 normal bone marrow cells in both normal and BCL$_1$ tumor-bearing mice. As judged by the number of colonies containing granulocytes (Table 1), the mice inoculated with leukemic B cells 4 weeks earlier provided an environment that enhanced proliferation and differentiation of CFU-DG compared with controls ($P < .01$). The number of erythroid colonies (CFU-DE) stayed unchanged (Table 1), which implied the absence of an effect on erythroid differentiation.

Effect of BCL$_1$ CM on CFU-DG. To determine whether BCL$_1$ cells themselves were producing a humoral factor acting on CFU-DG, we assayed CM obtained from in vitro cultures of BCL$_1$ cells from the spleen and peripheral blood of leukemic mice. These CM contained an activity that stimulated CFU-DG ($P < .01$) but not CFU-DE (Table 2).

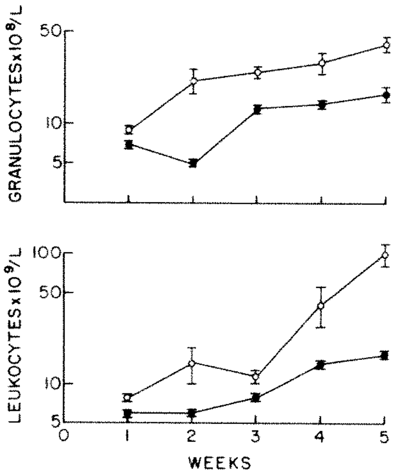

Fig 1. The number of leukocytes and granulocytes in normal BALB/c mice (●——●) and mice carrying the BCL_1 tumor (○——○) was determined weekly.

Table 2. Effect of Humoral Activities Derived From BCL_1 Mice on Colony Formation in Diffusion Chambers In Vivo

Type of Treatment	CFU-DG/Chamber* ± SEM	CFU-DE/Chamber ± SEM
Control	53 ± 9	0
BCL_1 spleen cell CM	93 ± 12	4 ± 1
BCL_1 peripheral blood cell CM	89 ± 5	0
BCL_1 cell CM†	144 ± 20	5 ± 2
1:2 dilution	112 ± 10	3 ± 1
1:4	94 ± 7	5 ± 2
1:8	75 ± 8	2 ± 1
1:16	56 ± 6	3 ± 2
Normal B cell CM‡	56 ± 6	4 ± 2

*Each point represents 18 to 24 observations from at least three different experiments.

†T cells and macrophages were removed from the cell preparation.

‡Concentration of the preparation tenfold did not have further effect on colony formation.

Treatment of BCL_1 CM with limulus amebocyte lysate did not reduce D-CSF activity (data not shown). In further experiments, T cells and macrophages were removed from spleen cell suspensions by using anti–Thy 1 plus complement and adherence to plastic, respectively; the effect of media conditioned in the presence of this purified B cell population was then assessed. As shown in Table 2, a dose-dependent increase in the number of the CFU-DG per chamber in treated mice could be observed. Medium conditioned in the presence of B lymphocytes from normal BALB/c mice failed to enhance colony formation (Table 2), thereby suggesting that neoplastic B cells were the source of D-CSF.

Effect of BCL_1 CM on CFU-MIX, BFU-E, and CFU-C in vitro. Because several T cell lines produce many hematopoietic activities detectable by in vitro culture[12,13] along with factors detectable by in vivo diffusion chamber culture, we evaluated BCL_1 CM for its action on other hematopoietic precursors. As shown in Table 3, even after a tenfold concentration, BCL_1 CM had no effect on either multipotential (CFU-MIX) or unipotential erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) precursors in vitro. The same CM did not potentiate or inhibit the stimulatory effect on these precursors by EL-4 CM (Table 3). The addition of normal mouse serum to in vitro cultures containing BCL_1 CM had no effect on colony numbers (data not shown); neither did the addition of serum from mice treated with BCL_1 CM for three days contain any activity stimulating colony formation in agar (CFU-GM) or methylcellulose (BFU-E, CFU-MIX, data not shown). There was no difference between normal sera and sera from BCL_1 mice in stimulating colony formation in vitro (data not shown).

Effect of BCL_1 CM on CFU-MIX, CFU-S, BFU-E, and CFU-C in vivo. To determine whether BCL_1 CM had an effect on other precursor cells in in vivo cultures in addition to CFU-DG, the contents of diffusion chambers were harvested four days after implantation and subcultured in semisolid medium in vitro. As shown in Table 4, the number of CFU-S, CFU-MIX, BFU-E, and CFU-C was significantly increased, which implied that BCL_1 CM act either directly or indirectly via a more primitive cell on these precursors.

Biochemical characterization of BCL_1 CM. Biochemical characterization of D-CSF demonstrated that it is relatively heat stable (Fig 2) and resistant to neuraminidase treatment (Table 5). On the contrary, protease treatment of D-CSF abolished its bioactivity (Table 5). Table 6 summarizes a representative purification (one of three) of D-CSF from 10 L serum-free BCL_1 CM. Because many cytokines are glycoproteins, we passed BCL_1 CM over lentil-lectin agarose. Essentially all of the D-CSF activity was bound to this matrix and could be eluated with α-methyl-D-mannoside (data not shown). The lentil-lectin eluate was concentrated and fractionated by gel filtration on an Ultrogel AcA44 column. As shown in Fig 3, material with D-CSF activity was found in a pool of fractions near the ovalbumin standard (molecular weight [mol wt] 43,000). Finally, by using reverse-phase HPLC, D-CSF activity was recovered in a

Table 1. Colony Formation in Diffusion Chambers Implanted in Normal and Leukemic (BCL_1) Mice

Host Mice	CFU-DG/Chamber* ± SEM	CFU-DE/Chamber ± SEM
Control	69 ± 17	1 ± 1
BCL_1	157 ± 20	3 ± 2

*Each point represents 18 observations from three different experiments.

Table 3. Effect of Lymphocyte-Derived Humoral Activities on Colony Formation In Vitro

Source of CM	CFU-GM/Plate* ± SEM	BFU-E/Plate ± SEM	CFU-MIX/Plate ± SEM
Control	0	41 ± 2	0
BCL_1 cell line	0	44 ± 1	0
EL 4 cell line	70 ± 2	82 ± 4	7 ± 1
EL 4 cell line + BCL_1 cell line	67 ± 3	82 ± 4	8 ± 1

*Each point represents the mean of nine observations from three experiments.

Table 4. Effect of BCL_1 CM on the Number of Colony-Forming Cells in Diffusion Chambers in Mice

Host Treatment	Number of Colony-Forming Cells/Chamber ± SEM			
	CFU-S	CFU-MIX	BFU-E	CFU-C
Control	22.2 ± 2.0	3.4 ± 1.6	11.2 ± 3.0	18.8 ± 3.2
BCL_1 CM	34.2 ± 2.0*	11.8 ± 2.8†	50.6 ± 3.8†	95.2 ± 7.0†

Mice were treated daily with 0.5 mL BCL CM or control medium. After four days' diffusion chamber treatment, the contents were removed, and the number of precursors was determined by subculture, as described in Materials and Methods.

*$P < .05$.

†$P < .01$.

fraction containing approximately 20 μg protein (Fig 4, Table 6).

Absence of adrenocorticotropic hormone in BCL_1 CM. Because some B cell lines are known to produce adrenocorticotropic hormone (ACTH)[25] and because the number of CFU-DG increases after methylprednisolone administration to the host mice,[26] the concentration of ACTH[25] was determined in BCL_1 CM. The failure to detect any increased levels of this hormone suggests that D-CSF is not identical to ACTH and that the stimulatory effect was not mediated by corticosteroids.

DISCUSSION

Results from this study suggest that the enhancement of CFU-DG colony formation in diffusion chambers implanted intraperitoneally in BCL_1 tumor-carrying mice (Table 1) was mediated by a humoral factor. It was most likely elaborated by tumor cells because an increase in the number of colonies could also be observed after the administration of medium conditioned in the presence of spleen and peripheral blood cells consisting mostly of malignant B cells (Table 2). Normal macrophages and lymphocytes present in the cell suspension were an unlikely source because removal of these

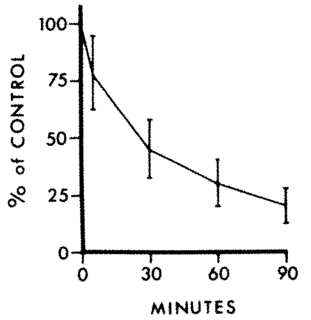

Fig 2. Heat stability curve for D-CSF present in BCL_1 CM.

Table 5. Effect of Trypsin, Pronase, Chymotrypsin, and Neuraminidase on D-CSF Activity

Treatment*	CFU-DG/Chamber ± SEM†
Control	71 ± 10
Trypsin	45 ± 4
Pronase	32 ± 5
Chymotrypsin	36 ± 3
Neuraminidase	67 ± 8

*All preparations were extensively dialyzed against PBS before administration to mice carrying diffusion chambers.

†Each point represents 18 points from three experiments.

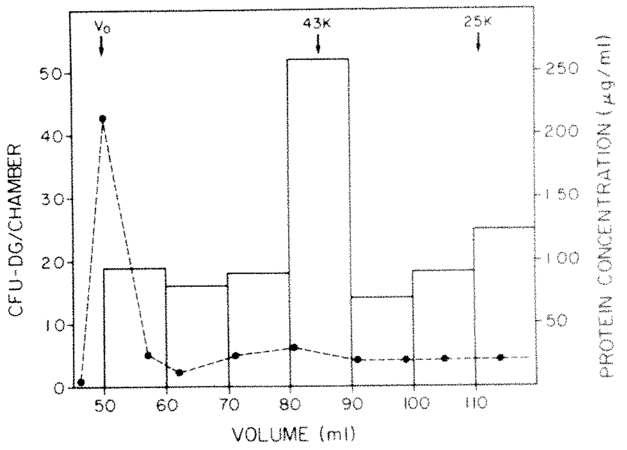

Fig 3. A representative illustration (one of five experiments) of partial purification of D-CSF. After tenfold concentration, BCL_1 CM was chromatographed on the lentil-lectin and eluted with α-methyl-D-mannoside. After another tenfold concentration, the material was then applied to an Ultrogel AcA44 column. Protein (----) was determined by the Bradford method.[24] The column was calibrated by using ovalbumin (43 kd) and chymotrypsinogen (25 kd).

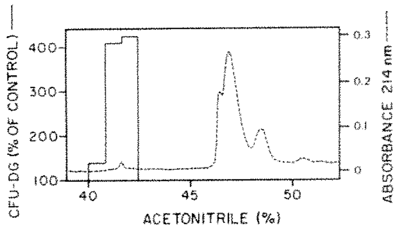

Fig 4. Reverse-phase HPLC elution profile. The material applied was prepared from 10 L serum-free BCL_1 CM through lentil-lectin and Ultrogel chromatography. The protein was eluted with acetonitrile and monitored by absorbance at 214 nm (----). Each 3-mL fraction collected by a FRAC-100 fraction collector was assessed for D-CSF activity.

Table 6. Purification of D-CSF From BCL_1 CM

Step	Protein (mg)	Approximate Activity (U)	Estimated Specific Activity U/mg*	Apparent Yield (%)
CM	360	936,000	2,600	
Lentil-lectin	19	307,800	16,200	32.9
Ultrogel	0.56	53,000	94,600	5.7
Reverse-phase HPLC	0.020	8,600	430,000	0.9

*Based on a dilution curve, the amount of protein giving rise to one colony above the number of colonies in the control was considered 1 unit.

cells did not diminish the potency of the conditioned medium (Table 2). Additionally, medium conditioned in the presence of B cells from normal BALB/c mice failed to stimulate CFU-DG (Table 2), which suggested that nontransformed B cells do not produce D-CSF or that B cells must be activated to produce D-CSF.

The failure to stimulate BFU-E, CFU-C, and CFU-MIX colony formation in vitro by BCL_1 CM (Table 3) confirms our previous results indicating that D-CSF from human sources, like murine D-CSF, is different from erythroid-potentiating activity (EPA) GM-CSF, and multi-CSF. By boiling and using chromatographic techniques we were able to separate human T cell line (Mo and HUT 102)-derived D-CSF from EPA, CSF, and multi-CSF.[12,13,27] Unlike human GM-CSF, human D-CSF,[13] like murine CSF (Fig 2), was relatively heat stable, and on diethylaminoethyl–Sepharose chromatography it had less affinity for the matrix than EPA and multi-CSF.

The increase in the number of CFU-S, CFU-MIX, BFU-E, and CFU-C in the diffusion chambers in addition to enhanced CFU-DG colony formation suggests that production of these hematopoietic precursors, at least in in vivo culture, can be modulated by a stimulatory activity present in BCL_1 CM that is different from multi-CSF, EPA, and GM-CSF. The failure of colony-forming cells to respond in vitro to BCL_1 CM (Table 3) may indicate that expression of its stimulatory effect on hematopoietic progenitors requires the presence of compounds occurring in vivo but not the ingredients of in vitro cultures. The lack of stimulation after the addition of mouse serum to these cultures (data not shown) does not exclude this possibility because conditions inside the diffusion chambers in the peritoneal cavity are different. Alternatively, BCL_1 CM acts on CFU-DG indirectly via another growth factor. The inability to detect increased levels of GM-CSF, EPA, or multi-CSF in the serum of mice carrying BCL_1 or BCL_1 CM–treated mice may indicate that these factors are not involved in the modulation of CFU-DG growth. However, it is possible that D-CSF acts locally on cells that are different from CFU-DG and induces them to elaborate growth factors acting on CFU-DG.

Unchanged hematocrit values and platelet counts in mice receiving BCL_1 CM were not unexpected, despite the possibility that CFU-DG may be at least as primitive as multipotential 12-day CFU-S. It has been shown that when lithium is administered to mice, regardless of its stimulatory effect on CFU-DG[28] and CFU-S,[29] only peripheral blood granulocyte counts are higher. The data in the prior study[28] suggested that lithium, like BCL_1 tumor cells, stimulates CFU-DG colony formation through a humoral factor that is different from CSF detected by in vitro CFU-C assay.

Involvement of GM-CSF in the development of granulocytosis secondary to neoplasms has been suggested by several clinical and animals studies.[30-37] However, the possibility that other factors cause granulocytosis cannot be excluded. A recent report describes poor correlation between levels of GM-CSF derived from two mammary adenocarcinoma tumor lines and granulocyte levels in host mice.[38] The results of this study suggest that an increase in the granulocyte count was mediated by D-CSF.

Traditionally, B lymphocytes have been thought to secrete only immunoglobulin, but recent reports indicate that these cells can produce other biologically active molecules, including growth factors,[39,40] endorphins and ACTH,[25,40] and B cell differentiation factors.[41] Our data demonstrate that neoplastic B cells produce, in addition to immunoglobulin, molecules capable of directly or indirectly stimulating hematopoietic precursors in vivo. Initial biochemical purification demonstrates that D-CSF resembles other hematopoietic factors.[42] Its attachment to a lentil-lectin column and its elution with α-methyl-D-mannoside suggests that D-CSF is a glycoprotein. Its mol wt (43,000) (Fig 3) is in the same range as that of other colony-stimulating factors,[41] and like EPA[43] in reverse-phase HPLC, it elutes with acetonitrile (Fig 4). It probably does not have ACTH-like biological activity because the ACTH concentration in BCL_1 CM was not elevated. This concern was prompted by the aforementioned report[25] indicating the production of ACTH by B cells and by our earlier study[26] indicating that corticosteriods exert a stimulatory effect on CFU-DG. The possibility that D-CSF represents a component of murine leukemia viruses also seems remote because the reverse transcriptase assay was negative.

These findings provide further evidence that the hematopoietic factor detected by in vivo diffusion chamber assay is different from factors detectable by conventional in vitro assay.

ACKNOWLEDGMENT

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Association of Hemoglobin H Disease With Hb J-Iran (β77 His → Asp): Impact on Subunit Assembly

By Samuel Rahbar and H. Franklin Bunn

A young Iranian female was found to be heterozygous for hemoglobin (Hb) J-Iran (β77 His → Asp) in combination with Hb H disease. The proportion of Hb J in the patient's hemolysate was suprisingly high: 65% Hb J, 30% Hb A. Thus, the interaction of a negatively charged β subunit variant of Hb with α-thalassemia leads to a marked increase in the relative amount of the variant Hb within red cells. This observation provides further support for an electrostatic model of Hb subunit assembly.

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THE DISTRIBUTION of normal and variant hemoglobins (Hbs) in heterozygotes provides insight into the biosynthesis and assembly of the Hb tetramer. Because one β globin gene is inherited from each parent, an individual heterozygous for a β globin variant would be expected to have approximately equal levels of normal and abnormal Hbs. A few β variants such as E, the Lepores, Knossos, K-Woolwich, and Vicksburg are synthesized at a significantly lower rate than βA. In heterozygotes these variants constitute much less than half of the total Hb and are associated with a thalassemic phenotype. Low levels of the variant are also observed in heterozygotes for unstable Hbs such as Köln and Zürich. The variability in levels of most of the remaining stable variants appears to be due to differences in the rate of subunit assembly.[1]

Hb J-Iran (β77 His → Asp) is one of the many informative Hb variants that have been discovered in the ethnically diverse population of Iran.[2,3] Subsequently this variant was reported in Turkey[4] and in a Russian-Armenian family.[5] The concentration of this variant in heterozygotes was always found to be nearly equal to that of Hb A. In this report we describe a 14-year-old female who had Hb J-Iran in combination with Hb H disease. Surprisingly, electrophoresis on both cellulose acetate and on citrate agar revealed twice as much Hb J as Hb A. This observation provides additional insights into how the surface charge on mutant Hb affects subunit assembly.

METHODS

Routine hematologic data were obtained by standard techniques. Erythrocytes were examined for inclusion bodies after treatment with brilliant cresyl blue. Hb stability was assessed by incubations of hemolysates in isopropanol.[6] Hb electrophoresis was performed on cellulose acetate (Tris-EDTA-borate buffer, pH 8.6) and on acid citrate agar gel.[7] Globin chain electrophoresis was also performed at both acid and alkaline pH.[7] The proportions of different Hb fractions were determined after cellulose acetate electrophoresis and elution of unstained Hb bands as previously described.[8] In vitro globin chain synthesis and α/β synthetic ratios were measured by standard techniques.[9] Structural identification of the variant Hb was established by isolation of the abnormal subunit by urea-carboxymethylcellulose chromatography,[10] digestion with trypsin, separation of tryptic peptides on two-dimensional maps, and amino acid analysis of abnormal peptides.[11]

RESULTS

A 14-year-old Persian girl was referred for investigation of mild jaundice and anemia. On physical examination her spleen was found to be moderately enlarged. Two other siblings were also found to have mild anemia and splenomegaly. Erythrocytes from the proband and from two of her siblings displayed characteristic inclusion bodies of Hb H disease. The proportion of red cells containing inclusion bodies was much less in the proband than that found in her two siblings. Nevertheless the isopropanol stability test was positive in all three individuals. Hb electrophoresis on cellulose acetate at alkaline pH confirmed the presence of Hb H in the proband as well as in these two siblings. In addition to Hb H, the proband showed a fast-moving Hb band in the position of Hb J. The distribution of Hbs in the proband's hemolysate was 65% Hb J, 30% Hb A, 3% Hb H, and 2% Hb A_2. The α-to-β synthetic ratio in the proband was 0.2, consistent with three-gene deletion α-thalassemia. Three other siblings were also heterozygous for Hb J but lacked Hb H. The variant in their red cells composed about 50% of the total Hb. The marked difference in Hb distribution between the proband and her siblings was confirmed by citrate agar electrophoresis at pH 6.5 as well as by electrophoresis and chromatography of globin chains. Structural analysis of the variant demonstrated that it was Hb J-Iran (β77 His → Asp), a variant frequently found among Iranians.

DISCUSSION

This is the first description of the association of Hb H disease with a negatively charged β subunit variant. This severe form of α-thalassemia imposes a marked increase in the proportion of the variant Hb in the circulating red cells (Fig 1). Previously, the association of another negatively charged variant, Hb J-Baltimore (β16 Gly → Asp), with probable homozygous α-thalassemia-2 (α-/α-) was noted to cause a moderate increase of the variant within the red cells.[12] In contrast, several reports have described the association of α-thalassemia (including Hb H disease) with positively charged β subunit variants such as Hb S, Hb C, Hb E, and Hb D.[13-15] In all cases the proportion of these variants

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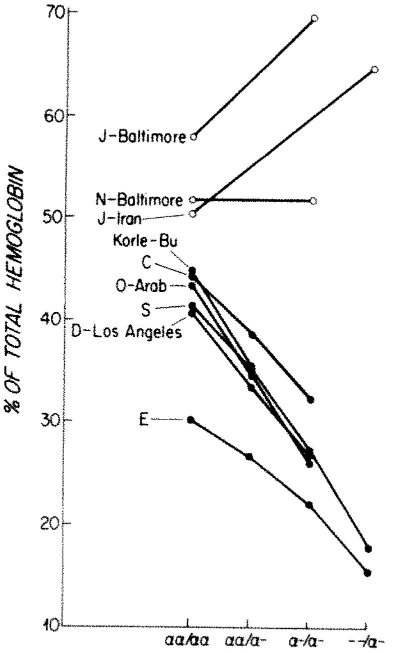

Fig 1. Effect of α-thalassemia on the proportion of negatively charged (○) and positively charged (●) β globin variants in heterozygotes. In all cases Hb A constitutes the great majority of the remaining Hb. In three-gene deletion α-thalassemia, a small amount of Hb H (β_4) is present. Modified with permission.[1]

in heterozygotes correlates inversely with the number of α genes deleted (Fig 1).

The substitution of an aspartic acid residue for a histidine in Hb J-Iran gives this variant a gain of 1.5 negative charge units. The higher negative charge on the surface of the βJ-Iran subunit would be expected to enhance its electrostatic attraction for the normal positively charged α subunit,[12,16,17] thereby allowing the formation of more Hb J-Iran than Hb A in the heterozygote's red cells. In the presence of α-thalassemia, the competition between βA and βJ subunits for the deficient α subunits is greatly enhanced, and even more Hb J-Iran is formed. The same mechanism applies to the observations noted earlier on Hb J-Baltimore. In fact, in vitro competition experiments using purified βA and βJ-Baltimore subunits have verified that when α subunits are deficient, the formation of Hb J exceeds that of Hb A by a factor of 1.5.[16] In contrast, the lower levels of the positively charged variants imposed by α-thalassemia can be explained by decreased electrostatic attraction between the α subunit and the variant β subunit.

In conclusion, the interaction of α-thalassemia with the negatively charged β globin variant Hb J-Iran provides informative and independent support for the importance of electrostatic attraction in the assembly of Hb subunits.[1]

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Granulocyte/Macrophage Progenitor Cells From Peripheral Blood and Bone Marrow Differ in Their Response to Prostaglandin E_1

By Carol M. Richman and George D. Johnson

Prostaglandins of the E series (PGE) inhibit proliferation of normal bone marrow granulocyte/macrophage progenitors (CFU-GM). Circulating CFU-GM are known to differ from marrow CFU-GM in many characteristics, and in the present study, we compared the effect of PGE_1 on circulating and bone marrow progenitors in normals and in patients with chronic myelogenous leukemia (CML). PGE_1 caused a dose-dependent inhibition of normal marrow CFU-GM. Circulating CFU-GM were inhibited only at concentrations of 10^{-5} mol/L or greater, and progenitor proliferation was, in fact, significantly stimulated at PGE_1 concentrations between 10^{-8} and 10^{-6} mol/L. Bone marrow CFU-GM from patients with CML were inhibited in a manner similar to that of normal bone marrow. Circulating cells from patients with CML were, however, less sensitive to PGE_1 inhibition than CML bone marrow cells and demonstrated a pattern intermediate between normal circulating and normal marrow progenitors. These studies suggest that peripheral blood and bone marrow contain different progenitor cell populations.

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PROLIFERATION OF GRANULOCYTE/macrophage progenitors (CFU-GM) in vitro is under the dual control of stimulatory glycoproteins collectively termed colony-stimulating activity (CSA)[1] and inhibitory substances such as prostaglandins of the E series (PGE) and acidic isoferritins.[2-9] Because these substances may also modulate hematopoiesis in vivo,[10-12] abnormal in vitro responses to these regulators have been sought to explain the pathogenesis of a variety of hematologic diseases. For example, insensitivity to PGE has been postulated as a mechanism for the excessive CFU-GM proliferation observed in chronic myelogenous leukemia (CML).[8,13-16] However, studies of prostaglandin regulation in myeloproliferative diseases, including CML, have often compared results obtained by using diseased circulating progenitors with results with normal bone marrow cells.[8,13,16] In normals, peripheral blood and bone marrow CFU-GM differ significantly,[17-24] and prior studies comparing normal marrow with abnormal blood progenitors have not fully explored these potential differences.

The present study was designed to compare the effects of PGE on circulating and marrow progenitors in normal individuals as well as in patients with CML. The results show that normal circulating CFU-GM proliferation is not decreased by prostaglandin E_1 (in physiological concentrations), but rather is significantly increased. PGE_1 does not appear to selectively increase a specific cytological colony subtype. Removal of adherent cells from the normal circulating cell population does not abolish the stimulatory effect if a source of CSA is provided. Bone marrow cells from both normals and patients with CML showed dose-dependent CFU-GM inhibition by PGE_1. Circulating CML cells (unlike normals) were not stimulated by PGE_1 but were less sensitive to PGE_1 inhibition than were CML marrow cells. These results, showing a difference in PGE_1 regulation between circulating and bone marrow CFU-GM, support the concept that the peripheral blood and marrow contain different progenitor cell populations. In addition, circulating CFU-GM from patients with CML respond to PGE_1 in a manner intermediate between the response of normal circulating and normal marrow progenitors.

METHODS

Normal bone marrow and peripheral blood samples were obtained from paid hematologically normal adult volunteers after written informed consent. Bone marrow and/or peripheral blood samples were obtained during routine clinical evaluations of 14 patients (nine males, five females) with Philadelphia chromosome–positive CML. Thirteen patients were in the stable chronic phase, and one had a chronic-phase blood picture (only the blood was studied) with evidence of early blast transformation in the bone marrow. The median age of the patients was 48 years (range, 26 to 72), the median leukocyte count at the time of study was 70×10^9/L (range, 14 to 136), and the median duration of diagnosis was 16 months (range, <1 to 70). Two patients had never been treated; 12 had previous therapy but no treatment for at least 2 weeks before the study.

Heparinized bone marrow aspirates and whole blood samples were separated on Ficoll-Hypaque density gradients (sp gr, 1.077). Mononuclear cells obtained from the interface were washed, depleted of adherent cells in some experiments,[25] and plated at final concentrations of 1×10^5 bone marrow cells/mL or 2 to 4×10^5 peripheral blood cells/mL in a methylcellulose culture system using leukocyte conditioned medium prepared with 1% (vol/vol) phytohemagglutinin (PHA-LCM) as the source of CSA.[26] The concentration of PHA-LCM used (10% of the total culture volume) provides maximal (plateau) stimulation of colony formation in this system. In some experiments, giant cell tumor conditioned medium (GCT-CM) was used as the source of CSA. PGE_1 (Sigma Chemical Co, St Louis) dissolved in ethanol was added to cultures at final concentrations of 10^{-10} to 10^{-4} mol/L. Indomethacin (Sigma) at 10^{-6} mol/L was added to all cultures to decrease endogenous PGE production.[2] Quadruplicate plates were incubated at 37°C in 10% CO_2 and high humidity for 14 days. Colonies (>20 cells) representing CFU-GM were counted by using an inverted microscope. Results were

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expressed as the percentage of control colonies: mean colonies with PGE$_1$ × 100/mean colonies without PGE$_1$. Statistical comparisons were performed by using Student's *t* test or paired *t* test. Fifty percent inhibitory doses (ID$_{50}$) were determined by linear regression analysis using a Texas Instruments calculator.

Cytological evaluation of single colonies was performed by removing all the individual colonies from methylcellulose plates with a drawn-out Pasteur pipette, placing each colony on a marked portion of a glass slide (two to three colonies per slide), and staining with Wright's stain. Each colony was classified as neutrophil, monocyte/macrophage, eosinophil, or mixed (neutrophil/macrophage) and the result expressed as the mean percentage of each colony type (± SEM).

RESULTS

PGE$_1$ effect on normal peripheral blood and bone marrow CFU-GM. A comparison of the effect of PGE$_1$ on normal bone marrow and peripheral blood CFU-GM is shown in Fig 1. At prostaglandin concentrations between 10^{-10} and 10^{-4} mol/L, dose-dependent inhibition of marrow CFU-GM was observed. Depletion of adherent bone marrow cells before the assay had no significant effect on PGE inhibition of bone marrow CFU-GM (data not shown). Peripheral blood CFU-GM were, in contrast, stimulated by PGE$_1$ in this concentration range, and results differed significantly from controls at PGE$_1$ concentrations of 10^{-8} and 10^{-6} mol/L ($P < .02$ and $P < .05$, respectively, paired *t* test). Maximal stimulation was achieved at 10^{-8} mol/L PGE$_1$; the response plateaued, and inhibition of circulating progenitors was observed only at PGE$_1$ concentrations exceeding 10^{-5} mol/L. The differences between the effects of PGE$_1$ on bone marrow and peripheral blood colony–forming cells were statistically significant for concentrations of 10^{-8}, 10^{-6}, and 5×10^{-6} mol/L ($P < .001$). Peripheral blood CFU-GM ID$_{50}$ occurred at a concentration of PGE$_1$ in excess of 10^{-5} mol/L, whereas the ID$_{50}$ for bone marrow cells is estimated to be 1.3×10^{-7} mol/L by linear regression analysis.

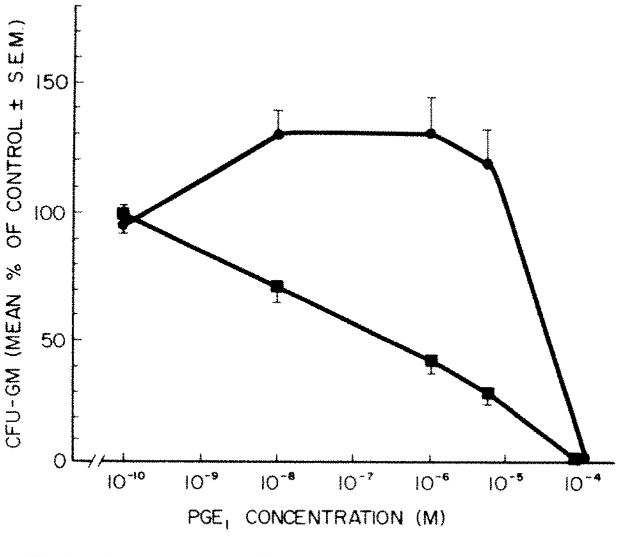

Fig 1. Peripheral blood (PB, n = 25, ●——●) and bone marrow (BM, n = 10, ■——■) cells from normal individuals were cultured with concentrations of PGE$_1$ from 10^{-10} mol/L to 10^{-4} mol/L using PHA-LCM CSA. Dose-dependent inhibition of BM CFU-GM was observed. The ID$_{50}$ determined from linear regression analysis is 1.3×10^{-7} M. PB CFU-GM were stimulated at all PGE$_1$ concentrations $< 10^{-5}$ mol/L. Stimulation is statistically significant at 10^{-8} and 10^{-6} mol/L ($P < .05$ and $P < .02$ respectively). The difference between PB and BM is statistically significant at 10^{-8}, 10^{-6}, 5×10^{-6}, and 10^{-5} mol/L PGE$_1$ ($P < .001, .001, .001, .05$).

To determine whether the type of CSA used to stimulate CFU-GM had any effect on the action of PGE$_1$, we compared PHA-LCM to GCT-CM by using 5×10^{-6} mol/L PGE$_1$. For all five normal bone marrow samples, PGE$_1$ caused greater inhibition of CFU-GM when GCT was used as a source of CSA compared with PHA-LCM ($P < .05$). However, normal circulating progenitors were not inhibited by 5×10^{-6} mol/L PGE$_1$ with either source of CSA.

Effect of CSA and adherent cells on PGE$_1$ stimulation of circulating normal CFU-GM. To evaluate the mechanism of stimulation of normal circulating progenitors by PGE, studies were performed with and without exogenous CSA. In ten experiments, the mean CFU-GM/4 × 10^5 cells was 26 ± 4 with CSA and 20 ± 6 without CSA, which suggested endogenous CSA production. In both CSA-stimulated and -unstimulated cultures, PGE$_1$ at concentrations of 10^{-8} and/or 10^{-6} mol/L significantly increased colony formation (Fig 2). Maximum colony formation occurred at 10^{-6} mol/L PGE$_1$, and the mean CFU-GM/4 × 10^5 cells were similar for cultures with and without exogenous CSA (40 ± 9 and 39 ± 8 respectively).

In ten experiments, nonadherent cells cultured with CSA (PHA-LCM) contained 34 ± 10 CFU-GM/4 × 10^5 cells compared with 1 ± 0 CFU-GM for nonadherent cells cultured without CSA (indicating depletion of endogenous CSA-producing cells). When nonadherent cells were plated with CSA and PGE$_1$, the results were similar to those using

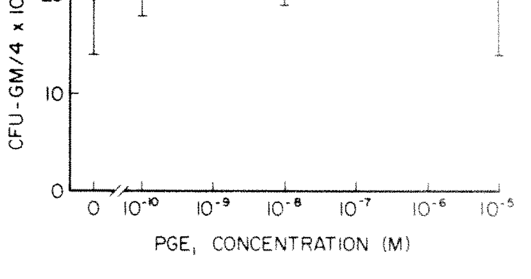

Fig 2. Normal peripheral blood mononuclear cells with (●——●) or without (O---O) exogenous CSA (n = 10) were cultured with PGE$_1$. CFU-GM/4 × 10^5 cells were increased by PGE$_1$ in CSA-stimulated ($P < .05$ at 10^{-6} mol/L PGE$_1$) and -unstimulated cultures ($P < .01$ at 10^{-8} mol/L, $p < .001$ at 10^{-6} mol/L and $P < .02$ at 10^{-6} mol/L).

whole mononuclear cells without adherence depletion, ie, PGE_1 at 10^{-6} mol/L caused significant enhancement of CFU-GM growth ($P < .05$).

In six of nine individuals studied, the maximum colony concentration in the nonadherent cells plated without an exogenous source of CSA was less than 5 colonies/4 × 10^5 cells with or without PGE_1. In one of the nine individuals, CFU-GM increased from 0 without PGE_1 to 6 with 10^{-6} mol/L PGE_1; in a second, CFU-GM increased from 0 to 12; and in a third, CFU-GM rose from 2 without to 51 with PGE_1.

PGE_1 effect on circulating and bone marrow CFU-GM from patients with CML. In patients with CML, CFU-GM from both the bone marrow and peripheral blood exceeded control values only at the lowest PGE_1 concentration (10^{-10} mol/L, Fig 3). At concentrations higher than 10^{-10} mol/L, there was a dose-dependent inhibition for both circulating and bone marrow cells. Unlike normal cells, circulating CML cells were not stimulated by PGE_1. However, blood CFU-GM were more resistant to PGE_1 inhibition than were bone marrow progenitors ($P < .05$ at 10^{-6} mol/L and $P < .01$ at 5×10^{-6} mol/L). For bone marrow, CFU-GM were 47% of control at 10^{-6} mol/L, whereas for circulating cells, values were still 58% of control at 5×10^{-6} mol/L. PGE inhibition curves for normal (Fig 1) and CML bone marrow (Fig 3) were not significantly different except at 10^{-10} mol/L PGE_1 where CML CFU-GM was 114% ± 6% of control whereas normal marrow CFU-GM was 97% ± 5% ($P < .05$). For normal and CML marrow cells, 50% inhibition is projected by linear regression analysis to occur at 1.3 and 4.1×10^{-7} mol/L respectively.

Cytological evaluation of PGE_1 effects. PGE_1 may inhibit macrophage colonies specifically,[4-6,27,28] although some reports suggest inhibition of both granulocyte and macrophage colonies.[3] To determine whether differences in the effects of PGE on circulating and bone marrow progenitors might be secondary to differences in the proportion of macrophage colonies, we characterized the type of colonies in control and PGE-exposed cultures. Individual Wright-stained colonies (see Methods) were analyzed from six peripheral blood and five bone marrow samples from normals (Table 1). There were significantly fewer macrophage colonies in the blood (19% ± 4%) than in bone marrow (38% ± 8%) ($P < .05$, paired t test). PGE_1-containing cultures of marrow had significantly fewer macrophage colonies than did marrow cultures without PGE_1 ($P < .05$). The distribution of cytological types of circulating or bone marrow colonies was otherwise not significantly affected by the addition of PGE_1.

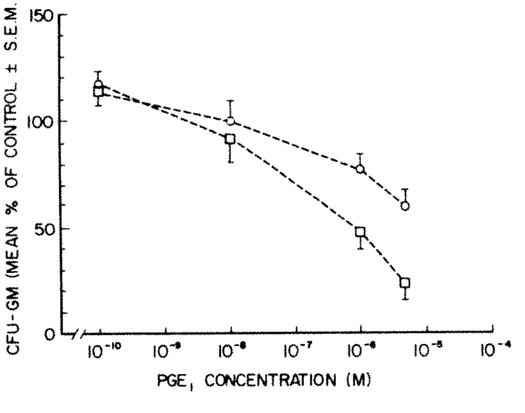

Fig 3. Peripheral blood (PB, n = 14, O---O) and bone marrow (BM, n = 9, □---□) from patients with CML were cultured with 10^{-10} to 5×10^{-6} mol/L PGE using PHA-LCM CSA. Dose-dependent inhibition of both PB and BM CFU-GM was observed at doses exceeding 10^{-10} mol/L. PB CFU-GM are more resistant to PGE_1 inhibition than BM CFU-GM. By linear regression analysis, the ID_{50} for PB is 8.1×10^{-6} mol/L, and for BM it is 4.1×10^{-7} mol/L.

DISCUSSION

Normal peripheral blood and bone marrow granulocyte/macrophage progenitors differ significantly in their response to PGE_1. The inhibition of marrow CFU-GM by PGE_1 observed in the present study is similar to that reported by others.[2,8,13,14,28] Peripheral blood CFU-GM were not inhibited by PGE_1 and were, unexpectedly, stimulated by concentrations in the range of 10^{-8} to 10^{-6} mol/L, an effect not previously reported for normal peripheral blood. PGE_1 augmented CFU-GM in peripheral blood cultures containing an exogenous source of CSA, either PHA-LCM or GCT medium, as well as in cultures stimulated only by endogenous CSA production. The stimulatory effect of PGE_1 on peripheral blood progenitors was not significantly affected by depletion of adherent cells, thereby suggesting that these cells do not solely mediate the stimulatory effect. Nonadherent cells showed negligible spontaneous CFU-GM growth, which indicated adequate depletion of endogenous CSA-producing cells. In six of nine individuals studied, PGE_1 had no effect on nonadherent cells in the absence of exogenous CSA. In the other three, however, PGE_1 stimulated CFU-GM in the nonadherent cell fraction.

Stimulatory effects of PGE on CFU-GM have been noted variably in studies of circulating progenitors in patients with myelofibrosis and myeloid metaplasia (MMM).[16,29] The subpopulation of progenitors in MMM that are stimulated by PGE may be similar to normal circulating progenitors. One

Table 1. Effect of PGE_1 on Cytological Type of Circulating and Bone Marrow Colonies

	No PGE_1	With PGE_1
Circulating colony type		
Neutrophil	43 ± 8*	40 ± 8
Monocyte/macrophage	19 ± 4	24 ± 5
Mixed	2 ± 2	4 ± 2
Eosinophil	36 ± 10	32 ± 10
Bone marrow colony type		
Neutrophil	36 ± 11	42 ± 7
Monocyte/macrophage	38 ± 8	16 ± 4†
Mixed	5 ± 1	10 ± 2
Eosinophil	21 ± 5	32 ± 7

*Percentage of colonies of a given type (mean ± SEM). Six blood samples and five bone marrow samples from normal individuals were studied.

†The difference between monocyte/macrophage colonies with and without PGE is significant at $P < .05$ when using the paired t-test.

might hypothesize that normal bone marrow contains primarily progenitors inhibited by PGE whereas normal blood contains CFU-GM stimulated by PGE and that the response of circulating CFU-GM to PGE would depend on the relative proportion of circulating marrowlike progenitors. Our results in CML showing effects of PGE on peripheral blood CFU-GM that are intermediate between normal blood and normal bone marrow CFU-GM support this notion because CML blood contains some cells that normally reside in the bone marrow. An alternative explanation is that circulating progenitors in these myeloproliferative diseases differ fundamentally from normal progenitors.

The cytological subtype of progenitors in peripheral blood and bone marrow differ, and our study is an agreement with other reports,[22] ie, fewer macrophage and greater numbers of eosinophil progenitors in the peripheral blood as compared with bone marrow. This difference could account for the resistance of circulating CFU-GM to inhibition by PGE if PGE$_1$ primarily inhibits macrophage colonies.[4-6,27,28] However, we observed not just a lack of inhibition with peripheral blood but a stimulation of CFU-GM, thereby suggesting that the presence of fewer committed macrophage progenitors in the peripheral blood does not totally explain the observed differences. Furthermore, cytological analysis of peripheral blood cultures with increased colonies in the presence of prostaglandin showed no consistent subtype of colony that was enhanced, which suggested an effect on an uncommitted CFU-GM.

The observation that circulating progenitors acquire some of the antigens present on marrow progenitors after short-term liquid culture with CSA has led to the suggestion that the blood CFU-GM are more primitive than bone marrow CFU-GM.[19,20] It has been proposed[23] that the situation is analogous to the more primitive erythroid precursor, BFU-E, found predominantly in the blood, and less primitive CFU-E, found predominantly in the marrow.[30] Subpopulations of CFU-GM have been observed in murine bone marrow with varying responses to PGE. Progenitors forming large colonies (assumed to be more primitive) are relatively resistant to PGE inhibition, whereas the more prevalent marrow CFU-GM form smaller colonies and are several orders of magnitude more sensitive to inhibition by PGE.[31] PGE in some reports appears to inhibit a subpopulation of CFU-GM that are in S phase and that express the Ia antigen.[32] Because normal human marrow and peripheral blood are known to differ in cell cycle status,[23] it is possible that such differences could account, in part, for the differences in PGE inhibition that we have observed.

CML bone marrow cells were, in the present study, as sensitive to PGE inhibition as were normal bone marrow cells. This result is in contrast to some reports indicating that CML progenitors from blood and/or bone marrow are extraordinarily resistant to PGE, with CFU-GM approximately 90% of control at PGE doses exceeding 10^{-6} mol/L.[8,13-16,29] Although we noted that CML peripheral blood progenitors were more resistant to PGE than bone marrow progenitors, even the blood CFU-GM were more easily inhibited in our studies than in the studies mentioned earlier. Another report found, as we did, no difference between normal and CML marrow in the degree of prostaglandin-induced inhibition.[33] Differences in culture technique may provide a partial explanation for these disparate results.

In the present study, normal peripheral blood and bone marrow CFU-GM differ significantly in their response to PGE$_1$. Circulating progenitors of patients with CML have PGE$_1$ responses that are intermediate between normal blood and bone marrow. If circulating progenitors represent a more primitive cell than their bone marrow counterparts, as has been suggested,[19,20,23,30] further studies of normal and pathological hematopoietic regulation by using circulating CFU-GM may supplement studies of bone marrow–derived progenitors.

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The Deletion in Both Common Types of Hereditary Persistence of Fetal Hemoglobin is Approximately 105 Kilobases

By Francis S. Collins, Jeffery L. Cole, Wendy K. Lockwood, and Michael C. Iannuzzi

The most common forms of hereditary persistence of fetal hemoglobin (HPFH) involve large deletions that remove the adult δ and β genes but leave the paired fetal genes ($^{G}\gamma$ and $^{A}\gamma$) intact. The size of these deletions has previously eluded exact definition. Using pulsed-field gel electrophoresis and the enzyme *Sfi*I, which cuts only rarely in genomic DNA, we have constructed a large-scale restriction map of the β-globin cluster in normal and HPFH DNA. The deletions in HPFH-1, which occurs in American blacks, and in HPFH-2, which occurs in Ghanaian blacks, are found to be approximately 105 kilobases (kb) in length, though the endpoints are staggered by approximately 5 kb. The fact that two previously reported γδβ-thalassemia deletions to the 5′ side of the β-globin cluster are also about 100 kb suggests a common mechanism, possibly involving the loss of a complete chromatin loop.

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HEREDITARY PERSISTENCE of fetal hemoglobin (HPFH) is a clinically benign and genetically heterogeneous condition characterized by continued expression of fetal hemoglobin (HbF) into adult life, with relatively balanced globin chain synthesis.[1-4] Several years ago, Southern blotting studies demonstrated that HPFH could be divided into two categories: those in which large genomic deletions removing the δ and β genes were present (deletion HPFH) and those in which the β-globin cluster appeared intact by Southern blotting (nondeletion HPFH).[5-7] In many of the nondeletion forms the overproduction of HbF is observed to be primarily $^{G}\gamma$ or $^{A}\gamma$, and recent cloning studies have identified point mutations in the promoter of the overexpressed fetal gene in most of these conditions.[8-14]

In deletion HPFH, however, there is usually overproduction of both $^{G}\gamma$ and $^{A}\gamma$ chains. The most common deletion type, denoted HPFH-1, occurs in American blacks and has a 5′ breakpoint approximately 4 kilobases (kb) 5′ to the δ gene (Fig 1).[5,7,15,16] HPFH-2, which occurs in Ghanaian blacks, has both its 5′ and 3′ endpoints shifted approximately 5 kb in the 5′ direction, but the total amount of DNA lost is within 1 kb of being the same as in HPFH-1.[6,7,15,16] Interestingly, homozygotes for HPFH-1 and HPFH-2 have no abnormality of phenotype other than their high level of HbF, which suggests that no other essential genes lie within the deletion.

Using cloned DNA from the breakpoints of these and other deletions, several laboratories have attempted to identify the total amount of DNA deleted in HPFH-1 and HPFH-2 by genomic blotting and walking studies. The presence of many repeated and recombinogenic sequences, however, has slowed this effort. Mager et al[17] recently concluded from their own walking efforts and a review of all available maps that the deletions must be at least 85 kb.

We have now applied the technique of pulsed-field gel electrophoresis (PFGE),[18-21] which allows the efficient separation of very large DNA molecules, to determine the actual size of these deletions and found them to be approximately 105 kb. This is remarkably close in size to the large deletions at the 5′ end of the β-globin cluster that cause γδβ-thalassemia, which adds further support to a hypothesis originally proposed by Vanin et al[16] that these deletions are not random but are generated by the loss of a complete chromatin loop.

MATERIALS AND METHODS

Cell lines. The source of HPFH-1 DNA was the cell line LAZ149, derived from a previously described and characterized individual homozygous for the HPFH-1 deletion[7] that was originally established by Dr Herb Lazarus and kindly provided by Dr Bernard Forget. HPFH-2 DNA was obtained from cell line GM2064 (available from the Mutant Cell Repository, Camden, NJ), derived from a homozygote for HPFH-2.[7] Another cell line from an HPFH homozygote, LAZ342, was obtained from Dr Stuart Orkin and was shown to be homozygous for HPFH-2 by Southern blotting (F. Collins, and J. Cole, unpublished observations) and by using the predicted fragment sizes in Table 1 of Tuan et al.[15] DNA representative of the normal β-globin cluster was prepared from the lymphoblastoid cell line 3.1.0.[22]

DNA preparation. DNA for use in PFGE analysis must have a very high molecular weight. Accordingly, we prepared DNA in agarose gel blocks basically as described by Smith et al and Collins et al.[23,24] In brief, cells were counted in a hemocytometer and then mixed with 2% low-melting–temperature (LMT) agarose (SeaPlaque, FMC Corp, Rockland, ME) and pipetted into a Lucite mold. The hardened blocks contained 2×10^5 to 2×10^6 cells in 80 μL and were then incubated for 36 hours in 500 mmol/L EDTA, 1% *N*-lauroylsarcosine, and 2 mg/mL proteinase K at 50°C to lyse the cells. Further incubations with 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, (TE), 1 mmol/L phenylmethylsulfonylfluoride, and TE were carried out to purify the DNA. Digestions were carried out for six to eight hours at 37°C or 50°C (*Sfi*I) by placing one block or a fraction of a block into an Eppendorf tube containing the appropriate restriction enzyme buffer in a total volume of 300 μL. We used 80 units of enzyme (all from New England Biolabs, Beverly, MA) for complete digests and 0.5 to 5 units for partial digests.

PFGE. Most gels were run by using the orthogonal field–alternation gel electrophoresis (OFAGE) modification of Schwartz and Cantor's original apparatus.[18,19] The principle involves alternation of two electric fields placed at approximately 90-degree angles.

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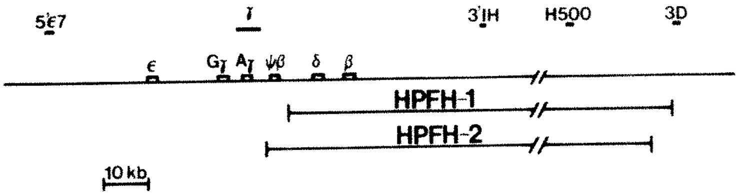

Fig 1. Map of the human β-globin cluster that indicates the limits of the HPFH-1 and -2 deletions. The total size of these deletions was previously undefined but known to be at least 85 kb. The probes 5'ε7, γ, 3'IH, H500, and 3D, which were used for this study, are described in the text.

A few gels were run on a commercially available PFGE apparatus (Pulsaphor, LKB Instruments, Houston). The running buffer was 0.4 × TBE (1 × TBE is 0.089 mol/L Tris base, 0.089 mol/L boric acid, 0.002 mol/L EDTA), and the gels were run at 14°C, 300 V, and 20 hours. Size markers were annealed λ DNA ladders, as previously described.[23] After ethidium bromide staining and photography, the gel was exposed to one minute of 254 nm UV irradiation to nick the DNA and then denatured and transferred to a nylon membrane (GeneScreen Plus, NEN, Boston) in 0.4 N NaOH.

Probes. The γ probe was a 3.5-kb *Hind*III fragment containing the entire $^{A}\gamma$ gene.[3] The 3D probe, kindly provided by Dr Bernard Forget, is a 1.0-kb *Bam*HI-*Eco*RI fragment derived from a genomic clone spanning the breakpoint in HPFH-1.[15] Probe 3D is located approximately 1 kb 3' to the end of this deletion. H500 is a 500-base pair (bp) *Hind*III fragment from a genomic clone kindly provided by Dr Dixie Mager that is located within the HPFH deletions, 9 kb upstream of the breakpoint of the Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thalassemia deletion.[17] H500 is therefore approximately 27 kb 5' to 3D and is derived from phage N3'β-1 as described by Mager et al.[17] The 3'IH probe is a 680-bp *Fok*I-*Bgl*II fragment isolated from near the breakpoint of the HPFH-3 deletion and is described by Henthorn et al.[25] The 5'ε7 probe is a 3,264-bp *Eco*RI-*Hind*III fragment located 16 kb 5' to the ε gene[26] and was supplied by Dr Oliver Smithies. All probes were prepared by electrophoresis of the cut plasmids in LMT agarose and labeling of the desired fragment to a specific activity of $>10^9$ cpm/μg by using the random hexanucleotide priming technique of Feinberg and Vogelstein.[27]

Hybridization. Prehybridization and hybridization were performed according to the membrane manufacturer's (GeneScreen Plus) recommendations. Blots were washed to a stringency of 0.1 × SSC (1 × SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate) at 65°C for 15 minutes and exposed against x-ray film with two intensifying screens for one to two days. As long as the blots were not allowed to dry, the probes could be stripped off and the blots rehybridized many times. Stripping was done by placing the blot in 0.4 N NaOH at 42°C for 30 minutes and then in 0.1 × SSC, 0.1% sodium dodecyl sulfate 0.2 mol/L Tris, pH 7.4, at 42°C for 30 minutes. Completeness of stripping was always assessed by exposure to x-ray film. A final hybridization was done with labelled λ DNA in most instances to define the size markers on the blot.

RESULTS

We performed genomic digests of lymphoblastoid cells from normal, homozygous HPFH-1, and two different homozygous HPFH-2 individuals by using a number of restriction enzymes that cut rarely in mammalian genomic DNA. These included *Mlu*I, *Bss*HII, *Not*I, *Pvu*I, *Sac*II, and *Sfi*I, which are all expected to produce fragments of 10 to 1,500 kb.[28] The DNA samples were prepared and restricted in agarose gel blocks, and then the DNA was electrophoresed by PFGE using annealed λ DNA ladders as a size marker (Fig 2). The DNA was transferred to a nylon membrane and hybridized. Only *Sfi*I gave detectable bands on the blot with any of the β-globin probes; all other enzymes gave a signal only at the top of the gel lane, which indicated a very large (>1,000 kb) fragment or gave no signal at all. The same blots rehybridized with nonglobin probes gave the expected single bands in these lanes (data not shown), thereby indicating that the DNA had been properly digested and transferred.

The results with *Sfi*I, however, were useful. This enzyme recognizes the sequence GGCCNNNNNGGCC and cuts

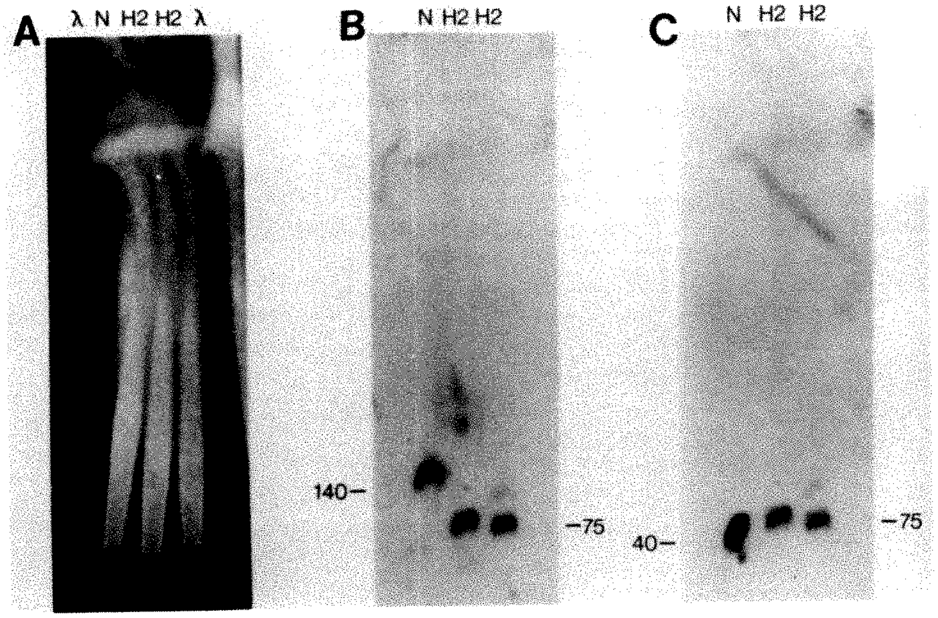

Fig 2. PFGE of normal and HPFH-2 DNA from two different patients. (A) Ethidium bromide stain of gel. The lane marked λ represents annealed λ DNA (genome size, 48.5 kb) used as a size marker. Complete *Sfi*I digests of normal (N) and HPFH-2 (H2) DNAs are shown. (B) Southern blot using the γ probe. (C) Rehybridization of the same blot with the 3D probe after stripping off the γ probe. The indicated sizes of the bands reflect corrections for the amount of DNA in each lane (see Fig 5). The H2 lane on the left is cell line GM2064; on the right is cell line LAZ342.

approximately every 250 kb in human genomic DNA. Unlike the other rare cutters tested, most *Sfi*I sites are not expected to be blocked by methylation because the recognition site does not include a CpG dinucleotide. Probing digests of normal and two different HPFH-2 DNAs (Fig 2) with an $^{A}\gamma$ sequence detects a 140-kb band for the normal and a 75-kb band for the HPFH-2 DNAs. This blot was then stripped, exposed to x-ray film to confirm complete removal of the first probe, and rehybridized with the 3D probe shown in Fig 1, which is just distal to the HPFH-1 breakpoint. The HPFH-2 DNA reveals the same 75-kb band, which is expected because $^{A}\gamma$ and 3D are only about 10 kb apart in the HPFH deletion. In normal DNA, however, a 40-kb *Sfi*I fragment is detected, thereby indicating that at least one *Sfi*I site lies within the deletion region.

It is possible to locate the 5′ end of the normal fragment containing the $^{A}\gamma$ gene exactly because there is a known *Sfi*I site approximately 17 kb 5′ to the ϵ gene for which an extensive 5′ flanking sequence was recently reported by Li et al.[26] Using a double digest of genomic DNA with *Eco*RI and *Sfi*I, we were able to show with a standard Southern blot that this *Sfi*I site cuts to completion in genomic DNA. Furthermore, a 675-bp *Ava*II-*Stu*I fragment of the 5′ϵ7 probe, located just upstream of this *Sfi*I site, hybridizes with a 10-kb *Sfi*I fragment (data not shown). No other *Sfi*I sites exist in the sequenced portion of the β-globin cluster.[3,26]

To complete the restriction map and define the size of the HPFH deletions, it was necessary to determine whether additional *Sfi*I fragments reside within the deletion region. The furthest available probe 3′ to the β gene, denoted 3′IH and derived from the breakpoint of the Indian type of HPFH (HPFH-3), has been determined to lie approximately 55 kb 3′ to the $^{A}\gamma$ gene[25] and therefore would be predicted to reside on the same 140-kb *Sfi*I fragment as $^{A}\gamma$. This was confirmed by hybridization of a PFGE blot (data not shown). From the other direction, Mager et al have cloned contiguous DNA sequences extending 30 kb 5′ to 3D.[17] Restriction digests of this cloned DNA revealed the presence of an *Sfi*I site approximately 19 kb 5′ to 3D (data not shown). A 500-bp *Hind*III fragment located 8 kb 5′ to this *Sfi*I site was gel purified; we denote this as H500. When this is used as a probe against normal and HPFH-2 DNA and the results compared with hybridization of the same blot with the γ probe, it is clear that H500 and γ are detecting the same 140-kb *Sfi*I fragment in normal DNA (Fig 3). That the fragment detected by H500 and γ is the same one and not a coincidental comigration of bands of equivalent size is further demonstrated by the identity of the larger bands, which result from the intentional partial digest used in this blot. The sizes of the partial-digest bands are readily explained by the position of the flanking *Sfi*I sites. As expected, H500 gives no signal from HPFH-2 DNA.

This allows the construction of a complete *Sfi*I map of the β-globin cluster and its 3′ flanking region (Fig 4). One can calculate the size of the HPFH-2 deletion as 140 + 40 − 75 = 105 kb. The calculation of these deletion sizes is quite sensitive to errors in the estimation of the PFGE blot bands. The use of λ concatemers allows interpolation of sizes to an accuracy of about 5 to 10 kb so long as the DNA fragments in the genomic digest lanes are migrating faithfully. We investigated the effect of the amount of DNA in the genomic digests on their electrophoretic mobility (Fig 5). The amount of DNA in a block is calculated from the number of cells used and the presence of 6.7 pg DNA/cell. We found that migration is consistent when the amount of DNA per lane is less than 2 to 3 μg. Above this amount, nonequilibrium effects in the overloaded lane result in a shift upward of the hybridizing band, thus leading to falsely large estimates of size. The size estimates shown in Fig 4 have all been determined by using 2 μg/lane or less of DNA. Allowing for a 5- to 10-kb error in the 40-kb fragment, and another 5- to 10-kb error in the difference between the 140- and 75-kb fragments (the difference is what enters the calculation), we would estimate that the measured HPFH-2 deletion size of 105 kb should be accurate to within approximately 10 to 20 kb.

Previous Southern blotting comparisons of the deletion endpoints in HPFH-1 and HPFH-2 have demonstrated that the total amount of deleted DNA is very similar,[6,7,15,16] with the HPFH-1 deletion being about 1 kb larger. Partial *Sfi*I

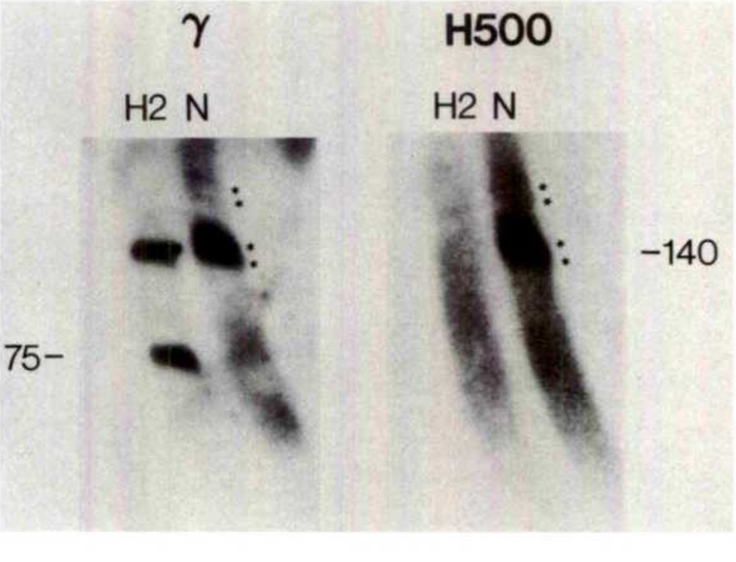

Fig 3. Partial *Sfi*I digests of normal (N) and HPFH-2 (H2) DNA probed with γ and then with the H500 probe that lies within the HPFH deletion. H500 and γ can be seen to detect the same *Sfi*I fragment in normal DNA, as evidenced by identity of the complete and partial digest bands (indicated by dots on the figure). The HPFH-2 DNA is from cell line LAZ342. The partial digest fragments in normal DNA represent approximately 150, 180, and 190 kb. The 140-kb partial digest band in HPFH-2 DNA indicates that the next *Sfi*I fragment to the 3′ side must be approximately 140 − 75 = 65 kb.

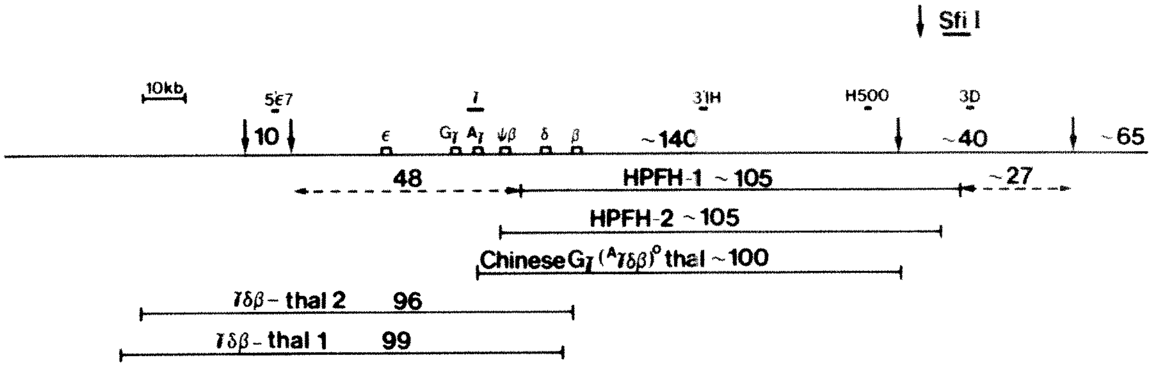

Fig 4. Map of the β-globin cluster. The vertical arrows mark the location of *Sfi*I sites. The sizes of *Sfi*I fragments and genomic deletions are given in kilobases. Note the similarity in size of the five deletions.

digests of HPFH-1 and HPFH-2 DNA were separated by OFAGE, blotted, and probed with the γ and 3D probes (data not shown). The fragments were indistinguishable, as expected because the 1-kb difference would not be resolved with this approach.

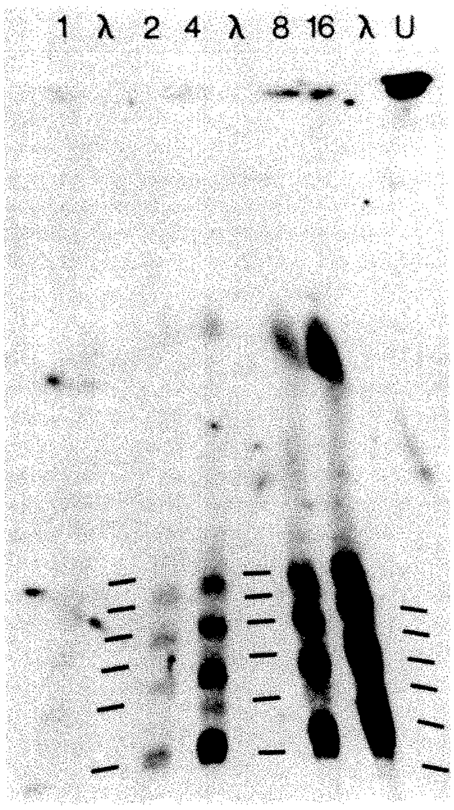

Fig 5. The effect of the amount of DNA per lane on the sizing of restriction fragments by OFAGE. Partial *Sfi*I digests of increasing amounts of normal DNA (1 to 16 μg, as indicated above the lanes and calculated from the number of cells embedded in the block) were electrophoresed in an OFAGE apparatus. Southern blotting and probing with the 3D probe shows that the size of fragments tends to be overestimated if more than 2 to 3 μg DNA is used. (The position of annealed λ DNA markers, genome size 48.5 kb, is indicated by bands drawn on the photograph.) The pattern of *Sfi*I partial-digest bands is entirely consistent with the map in Fig 4, which would predict bands of 40, 105, 180, and 245 kb.

DISCUSSION

The recent introduction of PFGE combined with enzymes that cut only rarely in genomic DNA allows the characterization of large regions that previously were relatively inaccessible to molecular biologic analysis.[28] This technique provides a useful bridge between standard molecular genetic approaches (which are difficult to extend to sizes greater than 50 kb) and cytogenetic analysis (which does not provide resolution below about 2,000 kb). The PFGE blotting approach has recently been applied successfully to the region of the Duchenne muscular dystrophy gene[29-32] and to the human major histocompatibility complex.[22,23] Here we have shown the application of this technology to the human β-globin complex. An *Sfi*I restriction map of the β-globin complex and its 3' flanking region has been constructed and used to determine the size of the HPFH-1 and HPFH-2 deletions without the need for time-consuming and difficult chromosome walking experiments. The map constructed here should be quite useful for analyzing other deletions.

The experiments reported here demonstrate that the deletions in HPFH-1 and HPFH-2 are approximately 105 kb. A third deletion, Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thalassemia, has previously been shown to involve the loss of a similar length of DNA as HPFH-1 (about 6 kb less) but with the endpoints shifted relative to the HPFH deletions (Fig 4).[16,17] Thus we can conclude that the Chinese deletion is approximately 100 kb in length. In the 5' direction, there are two other previously described large deletions for which the endpoints have been mapped, both of which give rise to $\gamma\delta\beta$-thalassemia. $\gamma\delta\beta$-Thalassemia 1 was described by Van der Ploeg et al[33] and has its 3' breakpoint 2.5 kb 5' to the β-globin gene, and $\gamma\delta\beta$-thalassemia 2 was subsequently shown by Orkin et al[34] to have its 3' breakpoint located within the β-globin gene. By cloning DNA located at the 5' breakpoints of these forms of $\gamma\delta\beta$-thalassemia, Vanin et al[16] were able to demonstrate that both of these mutations were simple deletions and that the total amount of deleted DNA was very similar. The absolute size of the $\gamma\delta\beta$-thalassemia deletions was subsequently determined by Taramelli et al[35] who performed a cosmid chromosomal walk crossing the entire region of these two deletions and demonstrated that they were 99.4 and 95.9 kb respectively. All five of these deletions, therefore, have removed 100 ± 5 kb genomic DNA, as shown in Fig 4.

Vanin et al,[16] noting that the $\gamma\delta\beta$-thalassemia deletions were of similar but at that time unknown size and that the HPFH deletions were also of similar but unknown size, have suggested a mechanism involving the loss of a complete chromosomal loop during DNA replication. What we have shown in this work is that the 5′ and 3′ deletions are all of approximately the same size, namely, about 100 kb. The model that we propose to explain this phenomenon, which is similar to that previously proposed, is built upon the following observations: (*a*) all of these deletions join nonhomologous DNA sequences,[16] which mitigates against a simple unequal crossing-over mechanism; (*b*) an accumulating body of evidence suggests that DNA is anchored to the nuclear matrix in vivo and is arranged in loops of length 30 to 130 kb[36-38]; (*c*) a simple loss of a chromosomal loop between fixed attachment points would not account for several staggered deletions unless DNA is capable of reeling through the anchorage points; (*d*) this reeling phenomenon may well occur during DNA replication because evidence suggests that DNA replication and topoisomerase activity is concentrated in the matrix[37]; and (*e*) DNA replication is bidirectional in prokaryotes and is probably so in eukaryotes.[37,39,40] In Fig 6 a possible mechanism for the generation of these deletions is depicted. Two matrix attachment points, M_1 and M_2, are postulated to be located physically close together and generate a loop of 100 kb containing the expressed genes of the β-globin cluster. During DNA replication, DNA is reeled into these attachment points from both directions, and daughter strands are reeled out. So long as replication proceeds at the same rate at M_1 and M_2, a faulty connection of the M_1 strand to the M_2 strand (with breaks occurring at M_1A and M_2A or M_1B and M_2B, as marked by arrows in the

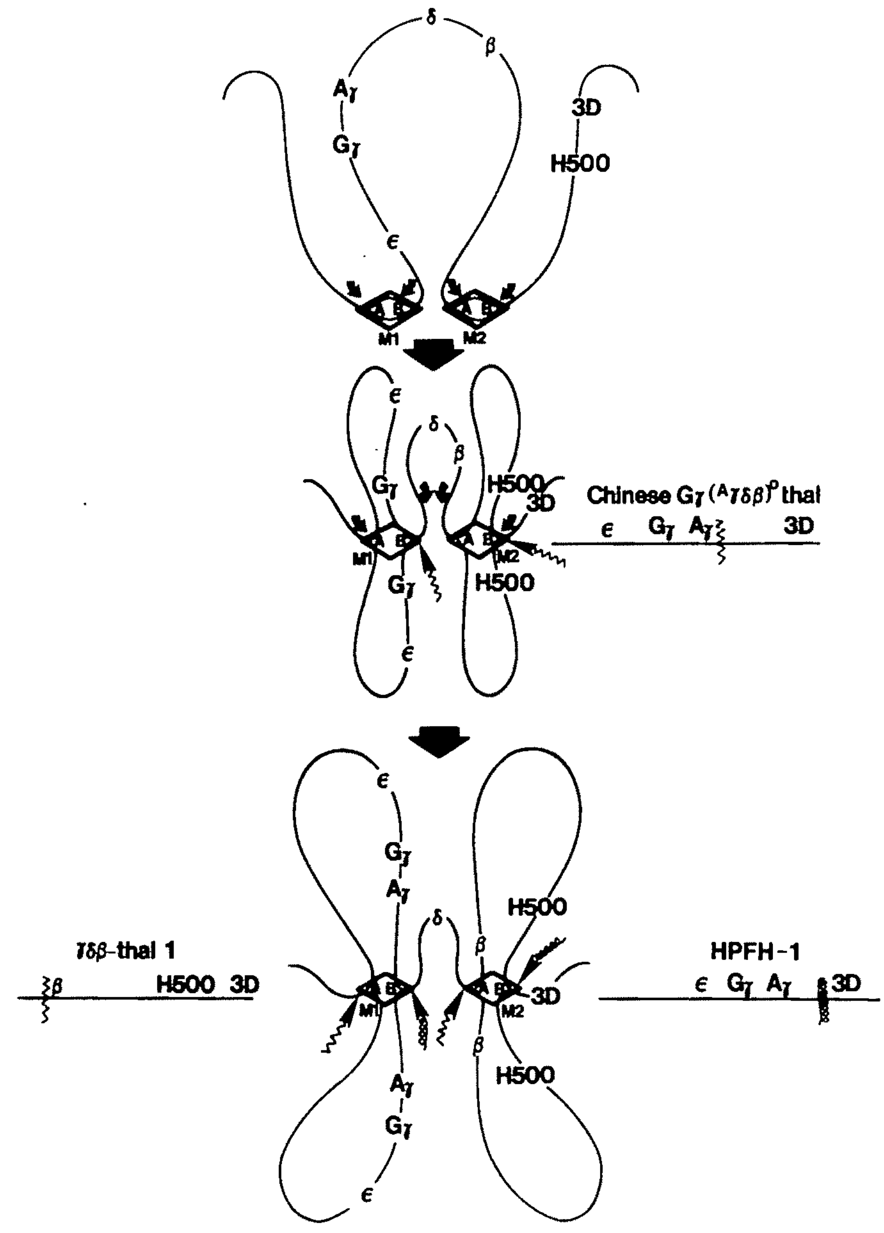

Fig 6. Postulated mechanism for generation of this series of β-globin deletions. M_1 and M_2 are two adjacent matrix attachment points. DNA replication occurs in a bidirectional fashion from these points, which leads to reeling in (heavy arrows) of DNA. Breaks at analogous positions on M_1 and M_2 followed by rejoining would then lead to deletions corresponding to the size of the original loop. Thus, in the middle panel, breakage at arrows marked ∿∿∿ would lead to Chinese $^{G}\gamma(^{A}\gamma\delta\beta)^{\circ}$-thalassemia, and in the lower panel, arrows with ∿∿∿ will lead to $\gamma\delta\beta$-thalassemia 1 and those with ℓℓℓ, to HPFH-1. HPFH-2 and $\gamma\delta\beta$-thalassemia 2 can be accounted for by the same model.

figure) will result in the deletion of 100 kb. Examples are shown in Fig 6. Smaller or larger (up to 200 kb) deletions of varying size would be generated if breakage and rejoining occurred between the A and B attachment points at M_1 and M_2. In fact, several smaller deletions in the β-globin cluster have been described, and at least one deletion has been reported that removes the entire cluster.[1] The model accounts for these but also predicts the observed series of staggered deletions of approximately 100 kb. Note that M_1 and M_2 must be located approximately as drawn in Fig 6 if this loop alone is to account for both the 5′ γδβ-thalassemia and the 3′ HPFH mutations. If adjacent loops are also of 100-kb size, however, the matrix attachment points could be located anywhere in this region.

If this model is correct, one would expect to observe similar phenomena in deletions at other loci, though the size of the deletions would be expected to vary from locus to locus according to the location of nuclear matrix attachment points. Interestingly, Nicholls et al have recently performed a detailed analysis of deletions in the human α-globin locus,[41] and a series of staggered deletions involving the loss of 20 to 30 kb has been identified.

An unexpected finding of this study was the inability to detect bands of measurable (<1,000 kb) size with a wide variety of restriction enzymes that ordinarily generate fragments of 100 to 1,000 kb in mammalian genomic DNA. All of the enzymes tested, except *Sfi*I, have recognition sites containing at least one CG dinucleotide, and current evidence suggests that cutting by these enzymes is blocked by methylation of the cytosines.[28] The ability to generate measurable restriction fragments with these enzymes in other parts of the genome is felt to be attributable at least in part to the presence of so-called *Hpa*II tiny fragment islands, which are highly GC rich sequences often found in the 5′ flanking region of housekeeping genes and usually unmethylated in this setting.[42] The fact that none of these methylation-sensitive enzymes would cut within 1,000 kb of the β-globin cluster suggests that the domain of DNA methylation around this region is very wide, at least in lymphoblastoid cells. In fact, there are *Mlu*I and *Bss*HII sites in sequenced DNA of the β-globin cluster,[3,26] and we have shown that in lymphoblastoid cells these sites do not cut at all in Southern blotting experiments (F. Collins and J. Cole, unpublished data). It would not be surprising if there are other regions of the human genome that are relatively deficient in restrictable methylation-sensitive sites, and this fact will have important consequences for general strategies to construct a large-scale restriction map of the genome.[28]

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Unique Interactions of Asialo von Willebrand Factor With Platelets in Platelet-Type von Willebrand Disease

By Jonathan L. Miller, Zaverio M. Ruggeri, and Vicki A. Lyle

The present studies demonstrate that platelets from patients with platelet-type von Willebrand disease show specific and saturable binding of asialo von Willebrand factor (AS-vWF) under conditions where such binding is not observed with normal platelets. Although specific binding of ^{125}I–AS-vWF to formalin-fixed normal platelets could not be demonstrated, specific binding to fixed patient platelets was seen with an apparent K_d of 1.3 μg/mL and specific maximally bound ligand of 0.40 μg/10^8 platelets. Preincubation of patient platelets with the antiglycoprotein Ib (anti-GPIb) monoclonal antibody AS-2 reduced total binding close to the level of computer-estimated nonspecific binding. In contrast, binding was not reduced by preincubation with anti-GPIIb/IIIa monoclonal antibody or with 5 mmol/L EDTA. Under stirring conditions, the binding of AS-vWF to fixed patient platelets was accompanied by a strong agglutination response. AS-vWF–induced agglutination was similarly observed in patient but not normal platelet-rich plasma (PRP) in the presence of 5 mmol/L EDTA. In the absence of EDTA, AS-vWF produced a full aggregation response in patient PRP at concentrations as low as 0.1 μg/mL in contrast to the 2 to 20 μg/mL required by normal PRP. Both thromboxane B_2 formation and adenosine triphosphate secretion showed an AS-vWF concentration dependence paralleling the aggregation responses. These studies show that a major difference in the platelets from patients with platelet-type von Willebrand disease is the presence of an exposed, high-affinity binding site associated with GPIb that recognizes AS-vWF.

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VON WILLEBRAND FACTOR (vWF) is a protein that plays a major role in platelet adhesive interactions. In an in vitro environment, platelets are agglutinated by vWF in the presence of the antibiotic ristocetin. This agglutination is dependent upon vWF binding to the platelets[1] at the platelet glycoprotein Ib (GPIb) receptor.[2] The mechanism by which vWF may bind in vivo to GPIb, in an environment lacking in ristocetin, is still unknown. After stimulation of platelets by thrombin,[3] adenosine diphosphate[4] and other agonists,[5] vWF may additionally be demonstrated to bind at the GPIIb/IIIa receptor.[6,7]

In contrast to native vWF, desialylated or asialo vWF (AS-vWF) has been shown capable of inducing platelet aggregation in the absence of ristocetin.[8,9] Aggregation was associated with the binding of AS-vWF to platelets and required the presence of free divalent cations. More recently, De Marco et al[10] and Gralnick et al[11] have used monoclonal antibodies to demonstrate that AS-vWF initially binds at the GPIb locus in normal individuals. After this interaction, there is exposure of the GPIIb/IIIa fibrinogen receptor. AS-vWF additionally appears capable of binding to the GPIIb/IIIa receptor complex.

Patients with platelet-type von Willebrand disease exhibit an abnormality of vWF binding. Patient platelets bind vWF in the presence of significantly lower concentrations of ristocetin than are required by normal platelets.[12-14] Additionally, specific and saturable binding of native vWF by patient platelets has been demonstrated in the absence of ristocetin or any other platelet agonist, and native vWF induces aggregation of patient platelets.[15] In view of the fact that desialylation changes the characteristics of vWF binding to platelets, we have undertaken the present studies to explore the interactions of AS-vWF with the abnormal platelets from patients with platelet-type von Willebrand disease.

METHODS

Purification and desialylation of vWF. Native human vWF was purified from human cryoprecipitate (provided by the Greater Syracuse Chapter of the American National Red Cross) as previously described.[15] This vWF retained normal multimeric composition[16] and ristocetin cofactor activity.[17] The purified vWF in phosphate-buffered saline, pH 7.3, was then digested with proteinase-free neuraminidase from *Vibrio cholerae* (Calbiochem, La Jolla, CA) as described by De Marco et al[9] at a ratio of 0.07 units enzyme/mg vWF. After a four-hour digestion, passage over a Sepharose 4B-CL column and subsequent dialysis against 0.02 mol/L Tris, 0.15 N NaCl, pH 7.3, the sialic acid content of the digested protein as well as of native vWF was assayed by the method of Warren.[18] Desialylated vWF to be used in ligand binding studies was labeled with ^{125}I by the method of Fraker and Speck.[19]

Blood processing and platelet function studies. For studies in platelet-rich plasma (PRP), normal or patient blood was drawn into 1/10 vol of 3.8% sodium citrate and the PRP prepared by centrifugation at 900 g for 75 seconds at room temperature (22° to 25°C) as previously described.[15] Unless otherwise specified, for studies of formalin-fixed platelets, platelets were prepared from acid-citrate-dextrose–anticoagulated blood by the method of Macfarlane et al[17] with the modification that the platelets were incubated for two hours at room temperature with a final concentration of 1.4% formalin in the absence of sodium azide. After formalin fixation, the platelets were resuspended in imidazole-buffered saline, pH 7.3, containing no divalent cations. Patient and normal platelets were alternatively processed and formalin fixed for 48 hours exactly as described by Takahashi and Shibata[20] for a smaller number of experiments. For platelet function studies the platelet count was adjusted to 200,000/

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μL and the platelets stirred at 1,200 rpm, 37°C, in a Chronolog (Havertown, PA) lumiaggregometer. Platelet-poor plasma (PPP) was used as a blank in the lumiaggregometer. For quantitative assessment of the extent of aggregation, percent aggregation was defined as maximal increase in light transmittance × 100/(light transmittance$_{PPP}$ − light transmittance$_{PRP}$). For studies of platelet dense granule release in PRP, luciferin-luciferase (Sigma Chemical Co, St Louis, MO) was added at a final concentration of 4 mg/mL to monitor the release of adenosine triphosphate (ATP).[21] In some experiments EDTA was added at a final concentration of 5 mmol/L before the addition of platelet agonist. At the end of the aggregation run, the cuvette was removed from the lumiaggregometer and thromboxane production determined as previously described.[22]

Monoclonal antibodies. Monoclonal antibodies AP-1 (anti-GPIb) and AP-2 (anti-GPIIb/IIIa) were a kind gift of Dr Thomas Kunicki of the Blood Center of Southeastern Wisconsin, Milwaukee. Monoclonal antibody AS-2, produced in our laboratory, is directed against the glycocalicin portion of GPIb (J.L. Miller and K.O. Hustad, unpublished observations). Antibodies were purified from ascitic fluid by precipitation with ammonium sulfate followed by affinity purification on Affi-Gel Protein A (MAPS II Kit, Bio-Rad Laboratories, Richmond, CA).

Binding studies. For binding assays, 25 μL of varying dilutions of ^{125}I-AS-vWF was added to 100 μL of a 10^8 platelets/mL suspension of formalin-fixed platelets in imidazole-buffered saline, pH 7.3. After a 30-minute room temperature incubation under nonstirring conditions, the platelets were centrifuged through 20% sucrose containing 2% bovine serum albumin (BSA) as previously described[15] and the platelet-associated radioactivity counted. Analysis of binding constants and Scatchard analysis of binding data were performed with the LIGAND (Scafit) computer program,[23] FORTRAN version 2.3.10 for the IBM PC.

RESULTS

Neuraminidase digestion resulted in the removal of over 90% of the sialic acid content of native vWF (10 nmol sialic acid/mg AS-vWF as compared with 114 nmol/mg native protein). Desialylation did not alter the multimeric composition of the vWF, nor were changes in multimeric pattern seen after iodination (Fig 1). Ristocetin cofactor activity of AS-vWF (119 U/mg) assayed as long as 5 weeks after desialylation did not differ significantly from that of the native vWF from which it had been produced (117 U/mg). Ristocetin cofactor assay of ^{125}I-AS-vWF revealed no loss of activity with respect to the unlabeled protein by 1 week after iodination, over which period of time the binding studies with this ligand were performed.

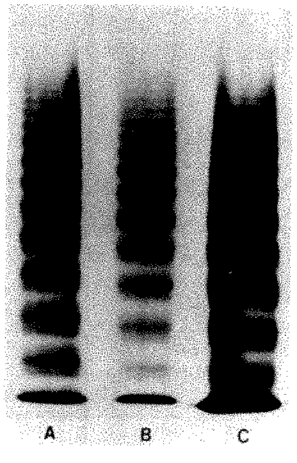

Fig 1. Sodium dodecyl sulfate agarose gel multimeric analysis of AS-vWF (B) and ^{125}I-AS-vWF (C) prepared, as described in the text, from the purified native vWF (A). The point of application is at the top, and the anode is at the bottom. A direct autoradiogram of the ^{125}I-AS-vWF run in lane C showed a similar multimeric pattern (data not shown).

When AS-vWF was added to the PRP from patients with platelet-type von Willebrand disease, a full aggregation response was seen at AS-vWF concentrations as low as 0.1 μg/mL (Fig 2). Increasing AS-vWF concentrations resulted in a more rapid attainment of this response. In contrast, with PRP from normal volunteers, an AS-vWF concentration of 2 to 20 μg/mL was required to achieve a full extent of aggregation. AS-vWF stimulation of thromboxane B_2 formation and of ATP secretion from platelet dense granules showed a concentration dependence very similar to that of the aggregation response both in patients and in normals (Fig 3).

The addition of 5 mmol/L EDTA to the PRP before the addition of AS-vWF fully blocked aggregation, thromboxane B_2 production, and ATP secretion with normal platelets. With patient platelets, in contrast, an initial change in light transmittance quite comparable to that observed in the absence of EDTA was seen after the addition of 2 μg/mL AS-vWF (Fig 2). This change in light transmittance in fact represented an agglutination of platelets rather than true aggregation, as evidenced by the lack of associated thromboxane formation or ATP secretion in the presence of the divalent cation chelator (Fig 3). At a lower concentration of AS-vWF (0.1 μg/mL) just sufficient to produce a full extent (but low initial rate) of aggregation in this patient, the prior addition of EDTA resulted in only a very marginal degree of agglutination (Fig 2).

After formalin fixation, platelet-type von Willebrand disease platelets suspended in medium containing vWF retained their abnormally increased sensitivity to low concentrations of ristocetin (Fig 4). As we have previously observed in the case of washed patient platelets suspended in modified Tyrode's buffer,[15] purified vWF added in the absence of ristocetin was incapable of agglutinating the fixed platelets. In the case of washed platelets, the addition of citrated PPP from a pool of normal donors or from the patients themselves

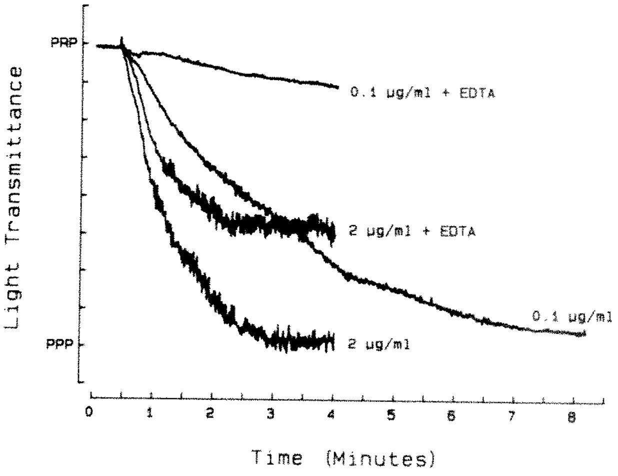

Fig 2. Effect of AS-vWF on citrated PRP from a patient with platelet-type von Willebrand disease. AS-vWF was added (upward deflection of trace) at the final concentrations indicated, in the absence or presence of 5 mmol/L EDTA.

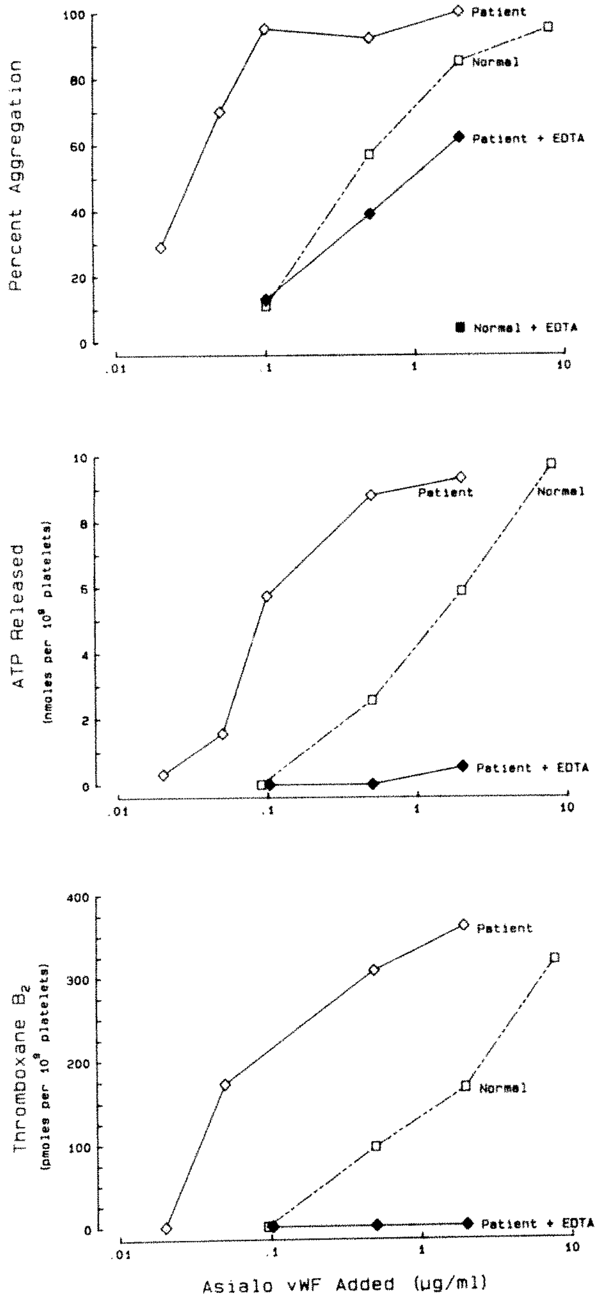

Fig 3. Platelet aggregation, ATP secretion, and thromboxane B_2 production in citrated PRP as a function of AS-vWF concentration in the absence (open symbols) or presence (solid symbols) of 5 mmol/L EDTA added before the AS-vWF. After completion of the lumiaggregation run, the cuvette was removed and thromboxane production subsequently determined.

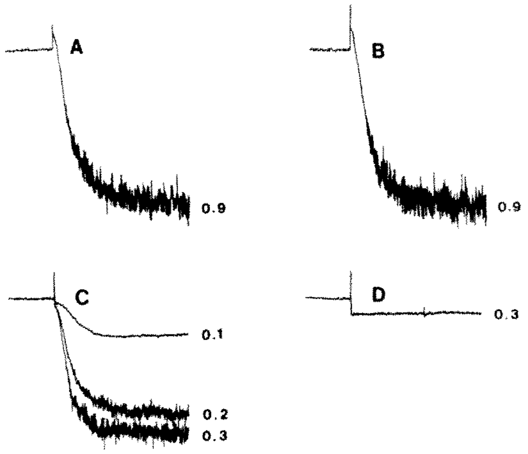

Fig 4. Agglutination of formalin-fixed patient platelets in the presence of native vWF (1.5 U/mL ristocetin cofactor, added as cryoprecipitate before the beginning of trace) and varying concentrations of ristocetin. Ristocetin additions are marked by an upward deflection of traces. The final concentration of ristocetin (mg/mL) is indicated at end of each trace. (A, C) Platelets from a patient with platelet-type von Willebrand disease. (B, D) Platelets from a normal control.

was capable of restoring responsiveness to vWF alone.[15] In contrast, the addition of an equal volume of PPP to a suspension of fixed patient or normal platelets produced no agglutination in the absence of ristocetin. The subsequent addition of either cryoprecipitate or purified native vWF (1 to 4 U/mL ristocetin cofactor activity) also failed to produce an agglutination response. Identical results were obtained when platelets were formalin fixed by the method used by Takahashi and Shibata.[20] Thus, after formalin fixation, the platelets of our patients with platelet-type von Willebrand disease appear to have lost the ability to be agglutinated by vWF in the absence of added ristocetin. They have, however, retained their increased responsiveness to quite low concentrations of ristocetin in comparison with fixed platelets obtained from normal donors.

In contrast to native vWF, the desialylated vWF produced a strong, concentration-dependent agglutination of the formalin-fixed patient platelets in the absence of added ristocetin. Normal platelets identically treated showed no agglutination response (Fig 5). Agglutination of the patient platelets was fully inhibited if the anti-GPIb antibodies AP-1 or AS-2 were added to the platelet suspension (25 to 40 µg/mL, final concentration) before the AS-vWF. Neither the anti-GPIIb/IIIa antibody AP-2 (40 µg/mL) nor the presence of EDTA (5 mol/L) inhibited this agglutination.

To distinguish whether the agglutination induced in patient platelets by AS-vWF was attributable to increased

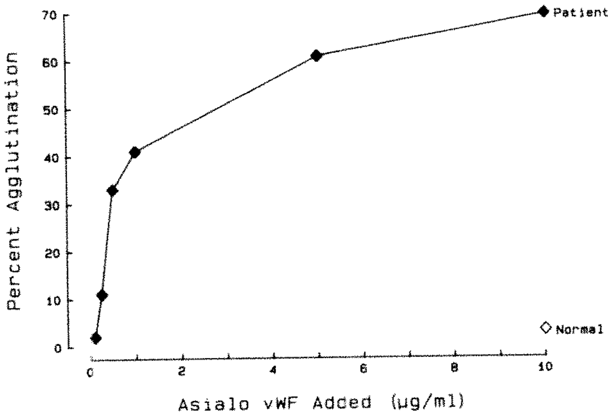

Fig 5. Agglutination of formalin-fixed platelets as a function of AS-vWF concentration.

AS-vWF binding or, instead, to a platelet abnormality subsequent to the initial binding step, we studied the binding of ^{125}I–AS-vWF to fixed platelets. Binding was performed at room temperature under nonstirring conditions. Analysis of the binding of ^{125}I-AS-vWF to patient platelets in the absence of any inhibitor (Fig 6, top panel, buffer condition) by the LIGAND[23] nonlinear curve fitting and Scatchard analysis program (Fig 7) produced estimates of an apparent dissociation constant of 1.3 μg/mL and specific maximally bound ligand of 0.40 μg/10^8 platelets. There was a relatively low residual variance (mean square) of 60 for this fit. The 0.8% nonspecific binding (0.26 μg/10^8 platelets at a 25-μg/mL ligand concentration) estimated by the program for the buffer condition was quite close to the actual binding observed when the platelets were preincubated with the anti-GPIb monoclonal antibody AS-2 (Fig 6, top panel). In contrast, neither the anti-GPIIb/IIIa monoclonal antibody AP-2 (Fig 6, top panel) nor the presence of 5 mmol/L EDTA (data not shown) produced any inhibition of binding. When fixed platelets from normal individuals were used in place of patient platelets in these studies (Fig 6, bottom panel, buffer condition), a specific binding component could not be identified by computer analysis. Moreover, no significant difference in binding to normal platelets was seen in the presence of AS-2 or AP-2, thus suggesting that the observed binding was in fact nonspecific in nature.

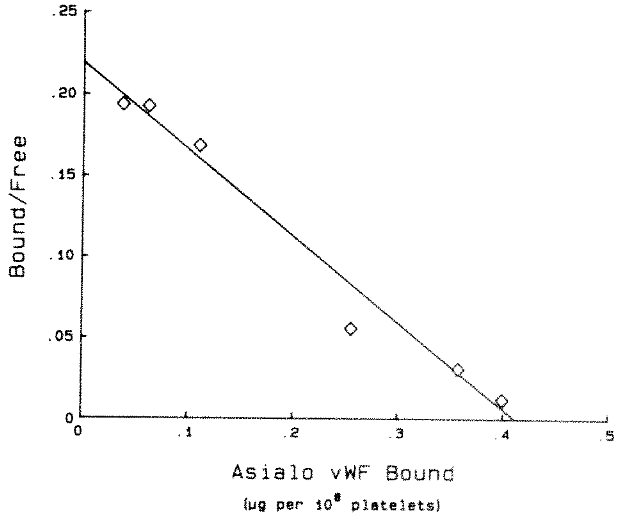

Fig 7. Scatchard plot of the same experiment for the buffer condition of patient platelets shown in Fig 6. Bound/free *v* bound (μg/10^8 platelets) represents the specific binding and is based on an estimate of 0.8% nonspecific binding by the LIGAND[23] computer program. Computer estimates of specific maximally bound ligand and of the dissociation constant for binding were 0.40 μg/10^8 platelets and 1.3 μg/mL, respectively, with a residual variance (mean square) of 60 for the fit.

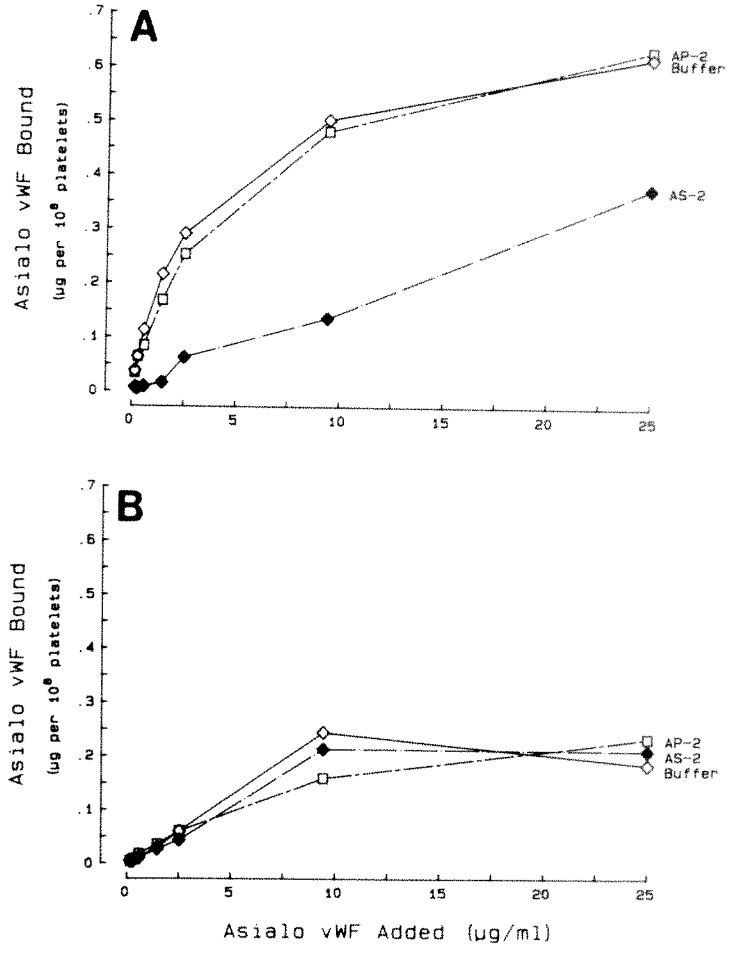

Fig 6. Binding of ^{125}I–AS-vWF to formalin-fixed platelets. Platelet suspensions were brought to a final concentration of 30 μg/mL antibody by adding to 100 μL of a 10^8 platelets/mL suspension of formalin-fixed (A) patient or (B) normal platelets 30 μL AS-2 (anti-GPIb), 30 μL AP-2 (anti-GPIIb/IIIa), or 30 μL buffer. After ten minutes at room temperature, 25 μL of varying concentrations of ^{125}I–AS-vWF was added to bring AS-vWF to the final concentrations indicated. After a further 30-minute incubation at room temperature under nonstirring conditions, the platelets were then centrifuged through a layer of 20% sucrose containing 2% BSA and the platelet-associated radioactivity counted.

When the binding of ^{125}I–AS-vWF to patient platelets was measured in the presence of >30-fold (wt/wt) excess of native vWF, the total amount of ^{125}I-AS-vWF bound was decreased by 20% to 25% (Table 1). This inhibition represented only approximately one third that achievable by the presence of anti-GPIb monoclonal antibody. Ristocetin greatly enhanced ^{125}I–AS-vWF binding to the patient platelets (Table 1). This enhancement was prevented if native vWF was present in the incubation mixture at the time of ristocetin addition.

DISCUSSION

The present studies have established that platelets from patients with platelet-type von Willebrand disease show specific and saturable binding of AS-vWF under conditions where such binding is not observed with normal platelets. Thus, binding of AS-vWF to platelets in the absence of ristocetin is demonstrable after formalin fixation of patient platelets, but not to formalin-fixed platelets from normal subjects. This binding is blocked by monoclonal antibody directed against platelet GPIb but not by antibody against GPIIb/IIIa or by the divalent cation chelator EDTA. This interaction now defines a third major ligand-binding abnormality of these platelets, following the prior demonstration of (*a*) enhanced ristocetin sensitivity for interaction of native vWF and (*b*) the binding of native vWF to metabolically active platelet-type von Willebrand disease platelets in the absence of any stimulating agent.[12,15] The present findings provide further evidence for an abnormality of the GPIb receptor complex in platelet-type von Willebrand disease.

The binding of AS-vWF to formalin-fixed patient platelets resulted in the agglutination of these platelets. Agglutination could also be demonstrated when AS-vWF was added to fresh patient PRP in the presence of 5 mmol/L EDTA. In contrast, as previously reported by other workers,[8,9] no agglutination of normal PRP could be demonstrated in the presence of EDTA. Under conditions where the platelets were metabolically active (PRP without added EDTA), thromboxane formation and dense granule secretion by patient platelets was observed at AS-vWF concentrations at least an order of magnitude lower than that required for normal platelets.

De Marco et al,[10] and Gralnick et al[11] have previously shown that in normal citrated PRP AS-vWF binds initially to GPIb and subsequently to the exposed GPIIb/IIIa receptor. The present studies indicate that in platelet-type von Willebrand disease platelets AS-vWF can bind to GPIb receptor sites even in the absence of free divalent cations. It is presumably the increased reactivity of these binding sites for AS-vWF in the patient platelets that leads in the presence of calcium ions to increased platelet activation, with subsequent thromboxane formation, dense granule secretion, and true aggregation. As in the case of normal platelets, the precise mechanism(s) by which binding at the GPIb site leads to platelet activation and to exposure of the GPIIb/IIIa receptor remains unknown.

The ability of formalin-fixed patient platelets to bind AS-vWF has permitted the study of AS-vWF binding restricted to the platelet GPIb receptor without the complexity of subsequent GPIIb/IIIa exposure that is seen in fresh platelets. Indeed, preincubation of the fixed patient platelets with the anti-GPIIb/IIIa antibody AP-2 (Fig 6) showed no diminution of the binding by AS-vWF. Even at concentrations as low as 0.4 to 1.0 μg/mL AS-vWF, significant binding to patient platelets was detected. This concentration range is quite similar to that at which a significant degree of AS-vWF–induced agglutination of patient platelets is detected. Thus, with AS-vWF concentrations for which approximately 0.1 to 0.5 μg of ligand are bound per 10^8 platelets, a strong agglutination response results. This is also very similar to the amount of AS-vWF that has been found necessary to bind to fresh normal platelets for an aggregation response to be observed.[9] It seems likely, therefore, that the major difference in the platelets from platelet-type von Willebrand disease patients is the increased affinity or more accessible binding of AS-vWF to the GPIb site. Once AS-vWF has bound to the required number of receptors per platelet, agglutination may ensue. Because fixed normal platelets are unable to bind comparable numbers of AS-vWF molecules, such agglutination would not be observed.

In the present studies native vWF appeared unable to produce an agglutination reaction in patient platelets in the absence of ristocetin. This finding is in contrast to the report by Takahashi and Shibata[20] where such an agglutination response was reported. It may well be that, although the patients with platelet-type von Willebrand disease in the present studies and those reported in the Japanese studies show major phenotypic similarities, the precise abnormalities at the vWF receptor region are not identical. Thus, although an abnormal band pattern of GPIb has been reported in the Japanese patients,[24] no GPIb structural abnormalities have yet been found in studies of this disorder by ourselves or others.[12,13] The qualitatively abnormal and high-affinity association of AS-vWF with the platelets from our patients with platelet-type von Willebrand disease may prove a useful

Table 1. AS-vWF Binding to Platelets From Patients With Platelet-Type von Willebrand Disease

Condition	^{125}I–AS-vWF Bound (Percentage of Control)
Buffer control	100
Anti-GPIb antibody AP-1 (40 μg/mL)	35.0 ± 6.1 (6)
Native vWF (44 μg/mL)	76.7 ± 4.1 (6)
Ristocetin (1 mg/mL)	585.2 ± 113.9 (6)
Native vWF (44 μg/mL) + ristocetin (1 mg/mL)	93.7 ± 12.9 (6)

A 25-μL aliquot of ^{125}I-labeled AS-vWF was added to 100 μL of a 10^8 platelets/mL suspension of formalin-fixed patient platelets in imidazole-buffered saline to bring the final concentration of AS-vWF to 1.2 μg/mL. After 30 minutes' incubation at room temperature under nonstirring conditions, the platelets were centrifuged through a layer of 20% sucrose containing 2% BSA and the platelet-associated radioactivity counted. Before addition of the ^{125}I-labeled AS-vWF, native vWF, ristocetin, the combination of both, anti-GPIb monoclonal antibody AP-1, or an equal volume of buffer was incubated with the platelet suspension for ten minutes at room temperature under nonstirring conditions. The total ^{125}I–AS-vWF bound under each experimental condition is expressed as a percentage of the mean total binding for the buffer control. Values shown are means ± SEM for (n) experiments.

tool for the identification of the underlying structural abnormality in these platelets.

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Inhibition of K Transport by Divalent Cations in Sickle Erythrocytes

By Carlo Brugnara and Daniel C. Tosteson

We report experiments on the effect of intracellular divalent cations (Mg, Ca, Mn) on K transport and cell volume in erythrocytes from patients with homozygous hemoglobin S disease (SS cells). When CO-treated SS erythrocytes are exposed to the ionophore A23187, removal of cell Mg markedly stimulates K efflux, whereas increasing cell Mg inhibits K efflux. The K_i for the inhibition by internal free Mg is 0.38 ± 0.10 mmol/L, a value comparable to the concentration of free Mg in normal cells (0.3 to 0.4 mmol/L). When swollen SS cells with increased Mg content were incubated in plasma-like medium, they shrunk much less than swollen SS cells with normal Mg content. Thus, elevation of cell Mg produces inhibition of swelling-induced K movement from SS cells. Internal Ca and Mn also inhibit K movement from SS cells. The inhibition of volume regulation by divalent cations suggests that increases in intracellular divalent ions, especially Mg, could induce a persistent degree of cell swelling in SS RBCs and thereby inhibit intracellular polymerization.

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POLYMERIZATION of hemoglobin S and cell sickling are extremely dependent on oxygen tension and hemoglobin concentration.[1] At any given oxygen tension, hemoglobin S concentration is the most important factor in determining the rate of polymer formation and morphological cell sickling. Because hemoglobin concentration is determined by the water and cation content of the RBC, it is important to study how water content and cell volume are regulated in sickle cell anemia. Cell volume is determined in RBCs by transport mechanisms moving cations and anions across the RBC membrane. We recently described a K transport system that is very active in oxygenated SS and CC cells[2,3] and is also present in the least dense (reticulocyte-rich) fraction of normal AA cells.[4,5] K movement through this system allows SS cells to shrink when they are isomotically swollen in vitro or when they are exposed to acid pH. This K movement is not sensitive to inhibitors of the Na-K pump (ouabain, 0.1 mmol/L) and/or Na-K-Cl cotransport (bumetanide, 0.01 mmol/L) systems, and differs from the Ca-activated K permeability first described by Gardos.[6] This pathway shares many properties with the pathway for K transport described by Lauf in sheep RBCs (SRBCs) (reviewed in ref. 7). A similar chloride-dependent pathway has also been described in "young" human RBCs by Hall and Ellory.[8]

Because cell swelling is a logical strategy to ameliorate the clinical course of SS disease,[9] and because this K transport pathway induces a sizable cation and water loss, thus counteracting cell swelling, and may also play a role in the formation of dense dehydrated SS cells, a detailed knowledge of its properties could be helpful in designing future pharmacologic interventions to induce cell swelling in SS cells.

The system for K transport described in low K (LK) SRBCs is modulated by divalent ions.[7] We studied the effect of divalent cations on K transport and cell volume in SS erythrocytes. We found that increasing internal Mg concentration in SS cells has marked effects on cell volume and K transport: An increase in internal Mg directly produces an increase of cell volume and thus a decrease of mean corpuscular hemoglobin concentration (MCHC). Moreover, the net K loss and cellular dehydration induced by acid pH- or swelling in SS cells are markedly inhibited by increased concentrations of internal Mg. Other divalent cations, such as Ca and Mn, share a similar inhibitory action, albeit with different affinities.

MATERIALS AND METHODS

Blood was drawn from patients homozygous for hemoglobin S disease. All patients had received no blood transfusions for at least 3 months prior to sampling. Preparation of RBCs, nystatin-loading procedure, and measurement of K efflux and phthalate density profile were performed as detailed in previous publications.[3] The methods we used to control and adjust Mg levels in erythrocytes and to measure total RBC magnesium with atomic absorption spectrometry were those described by Flatman and Lew.[10]

To study the effect of Mg on cell volume, the phthalate density profile was measured in SS cells incubated for 30 minutes in a medium containing 10 mmol/L NaCl, 130 mmol/L KCl, 0.5, 1, 5, and 10 mmol/L $MgCl_2$, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, and 90 μmol/L/cell A23187.

To study the effect of Mg, Ca, and Mn on K efflux, nystatin-swollen SS cells were incubated in isosmotic media containing 140 to 130 mmol/L choline chloride, 0 to 5 mmol/L divalents ($MgCl_2$, $CaCl_2$, $MnCl_2$), and 0 to 15 mmol/L sucrose. These three components were reciprocally varied to maintain constant chloride concentration and osmolarity. The media also contained 10 mmol/L Tris-MOPS, pH 7.40 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, 0.010 mmol/L carbocyanine[11] and 0.005 mmol/L chlorpromazine. Incubation times for K efflux measurement were 5 and 15 minutes. The incubation times in these and other experiments were chosen to meet conditions of initial rate and have K effluxes values linear with time. The hematocrit of the flux suspension was 1%, and the concentration of the ionophore A 23187 was 90 μmol/L/cell.

To study the effect of internal Mg, at constant external Mg, nystatin-treated SS cells were incubated in a medium containing 10 mmol/L NaCl, 130 mmol/L KCl, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, 90 μmol/L/cell A23187, and either 0.1 mmol/L EDTA or $MgCl_2$ (1 to 2 mmol/L). After 30-minute incubation, the ionophore was removed with four washes with the same medium plus albumin (1 mg/mL). K efflux was measured in a medium containing

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140 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-MES (pH 6.0 to 6.5) or Tris-MOPS (pH 6.75 to 8.0) at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, and 0.01 mmol/L bumetanide. The hematocrit of the flux suspension was 1%, and the incubation times were 5 and 25 minutes at 37°C (each one in triplicate).

To study the effect of internal Mg, at constant external Mg, on the volume regulatory decrease exhibited by swollen SS cells, a protocol similar to the one described above was followed and the cells were then incubated in a medium containing 140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L phosphate, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, and 10 mmol/L glucose. MCHC, Na, K, and Mg content and phthalate density profile were measured at the beginning of the incubation and after 12 hours at 37°C. Cell Mg and ATP contents were measured at the beginning and end of incubation and were constant.

RESULTS

Effect of divalent cations on cell volume in SS cells. As shown in Fig 1, when the internal concentration of Mg is increased by using A 23187 in SS cells, cell volume is also increased. This effect is due to the movement of chloride and thus water into the RBC, to balance the reduction in negative charges of nonpermeant solutes induced by Mg ions, and has been previously described in normal human RBCs by Flatman and Lew.[10] Cell swelling is not the only consequence of an increase on the internal Mg content: The increase of the chloride distribution ratio (Cl_i/Cl_o), at constant external pH and external chloride concentration, produces an alkaline shift in internal pH and a reduction of the RBC membrane potential. We also measured the total Mg content in SS and AA cells. Our preliminary data indicate that the total RBC Mg content is 2.5 ± 0.2 (SD, n = 5) mmol/L/cell in SS and 2.3 ± 0.1 (SD, n = 7) mmol/L/cell in AA cells (7.2 and 7.4 mmol/kg hemoglobin, respectively).

Effect of divalent cations on swelling-induced K efflux from SS cells. As in our previous study of CC cells,[3] we found no effect of external Mg on the swelling-induced K flux from SS cells (data not shown). As shown in Fig. 2a, however, when the *internal* Mg content is varied by varying the external Mg concentration in the presence of ionophore A 23187, K efflux from isosmotically swollen SS cells is markedly affected. Removal of internal Mg (by using A23187 and 0.1 mmol/L EDTA), stimulates K efflux from SS cells. When the internal (and external) Mg is increased, K efflux is progressively inhibited. At 0.15 to 0.2 mmol/L external Mg, K effluxes with and without A23187 are equal. This concentration of external Mg, with A23187, yields free and total RBC Mg values similar to those observed in fresh normal human RBCs[10] (C. Brugnara, unpublished observations). The K_i for external Mg, with A23187 added, calculated with the Dixon plot was 185 ± 50 μmol/L (n = 5). This value corresponds to a free internal Mg concentration of 380 ± 100 μmol/L.

To investigate whether other divalent cations have a similar inhibitory action on K transport of SS cells, we also increased the erythrocyte concentration of Ca and Mn. Because any increase of the internal free Ca opens Ca-activated K channels (Gardos effect, ref. 6), inducing a large K loss and also activating the Ca pump with associated increased consumption of ATP and metabolic substrates, we included in the incubation medium 10 μmol/L carbocyanine, an inhibitor of the Ca-activated K channel[11] and 5 μmol/L chlorpromazine, an inhibitor of the Ca pump. We did not use trifluoperazine because it produced cell lysis. The presence of these two drugs did not affect the inhibitory action of Mg on the OB-resistant K fluxes. Indeed, similar results were obtained when the internal Mg was varied with and without carbocyanine and chlorpromazine. Figure 2a shows the effect of external Ca and Mn, with A23187 added, on the swelling-induced K efflux from SS cells. When the internal (and external) concentration of these two divalent ions is increased, there is a marked inhibition of K efflux from SS cells. Mn is a more powerful inhibitor of K movement than Ca, since in two experiments the K_i for inhibition of K efflux by external Ca with A23187 were 300 μmol/L, and 200 μmol/L, whereas the comparable figures for external Mn with A23187 were 50 μmol/L and 70 μmol/L. Increasing concentrations of internal divalent cations inhibit the swelling-induced K efflux despite a concomitant increase in the cell water content that by itself would further stimulate K loss.

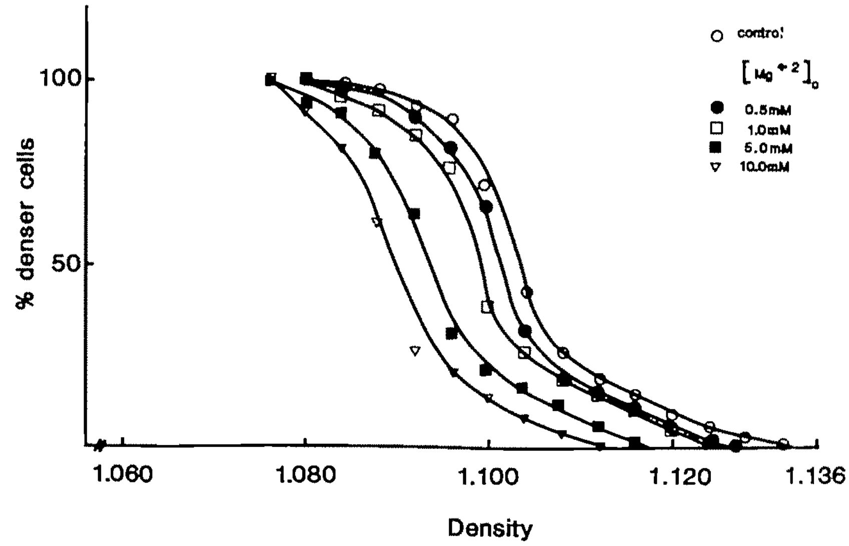

Fig 1. Effect of increasing the erythrocyte Mg content on density profile of SS cells. To increase cell Mg content, SS cells were incubated in a medium containing: 10 mmol/L NaCl; 130 mmol KCl; 0.5, 1, 5, and 10 mmol/L $MgCl_2$; 10 mmol/L Tris-MOPS, pH 7.4 at 37°C; 10 mmol/L glucose; 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, and 90 μmol/L/cell A23187. After 30-minute incubation at 37°C, the phthalate density profile was measured.

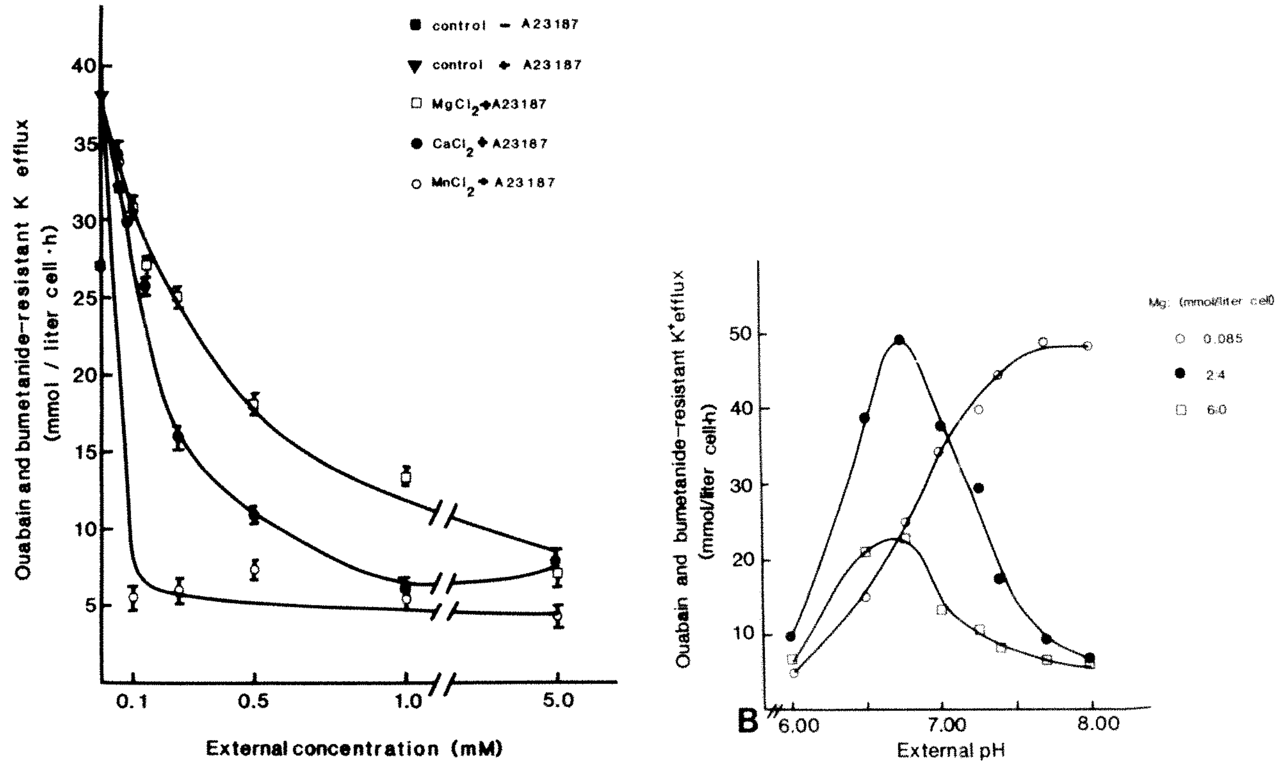

Fig 2. (A) Effect of divalent cations on the swelling-induced K efflux from SS cells. SS erythrocytes were isosmotically swollen by using the nystatin technique; MCHC values before and after nystatin treatment were 35.4 and 26.7 g/dL, respectively. K efflux was measured from cells in which divalent cations content was varied by using A23187 (90 μmol/L/cell) and different external divalent cation concentrations. The efflux media containing 140 to 130 mmol/L choline chloride, 0 to 5 mmol/L divalents ($MgCl_2$, $CaCl_2$, and $MnCl_2$), 0 to 15 mmol/L sucrose (these three components were reciprocally varied to maintain constant chloride concentration and osmolarity), 0.01 mmol/L EGTA, 10 mmol/L Tris-MOPS, pH 7.40 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, 10 μmol/L carbocyanine, and 5 μmol/L trifluoperazine. Incubation times for K^+ efflux measurement were 5 and 15 minutes, and the hematocrit of the flux suspension was 1%. (B) Dependence of ouabain- and bumetanide-resistant K efflux on external pH in control SS cells, and in SS cells with increased or decreased Mg content. MCHC, Na^+, and K^+ contents of nystatin-treated SS cells were 31.5 g/dL, 36.5 and 349 mmol/kg hemoglobin, respectively. The cell Mg content was varied with A23187, and the ionophore was then removed, as described in the Materials and Methods section. The total Mg content was 2.6 mmol/L/cell in SS control cells, 0.085 mmol/L/cell in SS cells with low Mg content, and 6 mmol/L cell in SS cells with high Mg content. K efflux was measured in a medium containing 140 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-MES (pH 6.0 to 6.5) or Tris-MOPS (pH 6.75 to 8.0) at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, and 0.01 mmol/L bumetanide. The hematocrit of the flux suspension was 1%, and the incubation times were 5 and 25 minutes at 37°C (each one in triplicate).

Effect of Internal Mg on the pH dependence of K efflux from SS cells. To rule out the possibility that these effects were due to A23187, and to measure K efflux from cells with different Mg content at constant (1 mmol/L) external Mg, SS cells were exposed to A23187 and different Mg concentrations, and the ionophore was then removed by washing at 37°C with albumin. When the RBC Mg content was increased, K efflux was markedly decreased at any external pH studied (Fig 2b). When the internal Mg was decreased (incubation in 0 Mg + 0.1 mmol/L EDTA), there was a marked change in the pH dependence of K efflux. K efflux was progressively stimulated when the external pH became more alkaline and the inhibition by alkaline pH was abolished (Fig 2b). Different effects were observed when cell volume was changed by altering the osmolarity of the medium at constant pH (7.40). At any given internal Mg concentration studied, K efflux was markedly inhibited when SS cells were shrunken by incubation in hypertonic media (data not shown). Thus, when the free and total cell Mg are markedly reduced in SS cells, there is a dissociation between the inhibitory action of alkaline pH (that is lost) and the inhibitory action of cell shrinkage (that is conserved) on K efflux.

Effect of internal Mg on regulation of cell volume by SS cells. We next studied the effect of varying the cell Mg content on the volume regulatory properties of CO-treated SS cells. SS erythrocytes were isosmotically swollen with the nystatin technique, their Mg content was increased with A23187 and MCHC, Na K contents and phthalate density distribution were measured before and after 12 hours incubation at 37°C in a plasma-like medium containing no transport inhibitor. As shown in Fig 3, swollen SS erythrocytes (total cell Mg 7.5 mmol/kg hemoglobin) shrink more than swollen SS cells with increased Mg content (total cell Mg 16.7 mmol/Kg hemoglobin). The difference in cell volume and cation content at the end of the incubation between SS control and SS cells with increased Mg content is mostly due to a smaller K loss in the high Mg cells (Table 1). There is

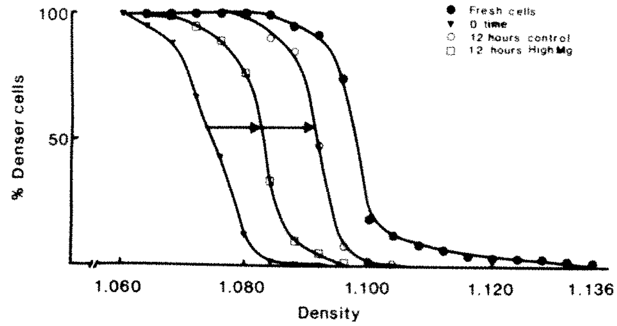

Fig 3. Effect of increasing the erythrocyte Mg content on phthalate density profile of swollen SS cells after 12-hour incubation in an isosmotic medium. The cell water and K contents were increased using the nystatin procedure. The erythrocyte Mg content was then increased by incubating the cells for 20 minutes with 90 µmol/L/cell A23187 in a solution containing 130 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris-MOPS, (pH 7.40) at 37°C, and 10 mmol/L glucose. The ionophore was then removed (as described in the Materials and Methods section), and the cells were then incubated for 12 hours in a medium containing 140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L phosphate, 10 mmol/L Tris-MOPS (pH 7.4) at 37°C, and 10 mmol/L glucose. This was one of three experiments in different SS subjects.

also a small increase of the cell Na content in the high Mg cells as compared with SS control cells.

DISCUSSION

This report shows that variations in cell Mg affect SS erythrocyte cell volume by two different mechanisms. The first mechanism occurs through a reduction of the net negative charge of the nonpermeant cellular ions due to Mg binding to organic phosphates (10). As a consequence, to maintain electroneutrality, chloride moves into the cells and water follows osmotically, increasing water content and volume[10] (Fig 1). The second mechanism involves a specific inhibitory effect of internal Mg on a K transport pathway that has increased activity in SS and CC cells,[2,3] but is also present in the least dense fraction of normal AA cells.[4,5] This inhibitory effect of Mg counteracts the cell shrinkage produced by this pathway when SS cells are swollen or exposed to acid pH (Figs 2B and 3).

Table 1. Effect of Increasing Total Cell Mg Content on Volume Regulatory Decrease of Swollen SS Cells

Cells	MCHC (g/dL)	Na	K	(Na + K)	Total Mg
		(mmol/kg hemoglobin)			
Fresh cells	35.2	44.3	240.0	284.3	7.4
Swollen SS cells					
0 time	26.7	30.4	475.0	505.4	7.5
12 hours	31.1	47.9	300.0	347.9	7.0
Swollen SS cells, high Mg content					
0 time	26.3	36.0	480.0	516.0	16.7
12 hours	28.4	53.5	361.0	414.5	15.0

Carbon monoxide-treated SS cells were isosmotically swollen by using the nystatin technique. The RBC Mg content was subsequently increased by exposing the RBCs for 20 minutes to 1 mmol/L $MgCl_2$ with 90 µmol/L/cell A23187, in a solution containing 130 mmol/L KCl, 10 mmol/L NaCl, 10 mmol/L Tris-MOPS (pH 7.40) at 37 °C, and 10 mmol/L glucose. After removal of the ionophore, the cells were incubated for 12 hours in a medium containing 140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L phosphate, 10 mmol/L Tris-MOPS (pH 7.40) at 37 °C, and 10 mmol/L glucose. This was one of three experiments in different SS subjects. Each determination was carried out in triplicate (SD 1% to 3%).

Other variables, namely internal chloride, internal pH, and membrane potential are changed when the internal concentration of divalent ions is increased. The inhibition is almost complete at a concentration of divalent cations in the external medium of 1 mmol/L (Fig 2A). This concentration of external Mg yields in the presence of A23187 a free cell Mg of 1.7 mmol/L (normal value is 0.3 mmol/L) and a total RBC Mg content of 6.5 mmol/L/cell water (normal value is 3.2 mmol/L/cell water). As a consequence, the internal chloride concentration rises, the internal pH increases from 7.25 to 7.29, and the membrane potential decreases from −9.0 to −6.5 mV. These last two changes by themselves could inhibit K movement and this possibility cannot yet be ruled out. The different affinities for divalent cations to inhibit K movement (Fig 2A) suggest, however, that this effect is a specific property of divalent cations rather than a secondary effect due to changes in internal pH and membrane potential.

The regulatory action of internal Mg on K movement is further revealed by the finding that decreasing internal Mg in human SS cells stimulates K efflux (Fig 2A and B). A similar effect has also been reported in LK SRBCs,[7,12] and the activity of the KCl cotransporter has been proposed to be determined by a divalent ion binding site, which is in turn regulated by the degree of protonation of its chemical groups.[7] Indeed, the stimulation of K efflux by removal of internal Mg is also associated with a loss of the bell-shaped dependence on pH of K movement, and K efflux is not inhibited when the internal pH becomes more alkaline (Fig 2B). Thus, the inhibition of this pathway for K transport requires the presence of internal Mg. It is reasonable to suppose that the inhibition of K transport by alkaline pH in the presence of internal Mg (Fig 2B) is due to binding of Mg at some site in the transport system for which Mg and protons compete. A loss of inhibition of K transport by alkalinization is also observed in SS, CC, and AA least dense cells after treatment with *N*-ethylmaleimide (NEM 1 mmol/L) or after isosmotic cell swelling[4] (data not shown). The only difference among these three different conditions is that the increased K efflux due to NEM treatment is not volume sensitive, whereas that due to Mg removal or isosmotic cell swelling is inhibited by cell shrinkage produced by suspension of the cells in hypertonic media.

We have proposed that the high activity of this pathway in CC and SS cells is due not only to the presence of young cells, but also to a direct effect of the positively charged S and C hemoglobins, probably through binding to the RBC membrane.[2-4] Indeed, cells with a moderate reduction of life span, such as those homozygous or heterozygous for C hemoglobin, have a much higher volume and pH-dependent K transport that control AA cells with comparable degrees of reticulocytosis. Although this pathway is active only in the least dense, reticulocyte-rich fraction of normal AA blood, which represents 5% of the whole RBC population,[4,8] it is active in all density fractions of CC and SS cells. Moreover, the K, Cl, and water loss induced by the activation of this transport pathway are not limited to a fraction of young SS cells, since they are easily demonstrable in whole SS blood (Fig 3 and Table 1). Because KCl loss through this pathway produces cell shrinkage, and because this pathway is most active in cells with relatively low density (and thus high cation and water content), it is reasonable to suppose that this pathway is involved in the reduction of K and water content that occurs when normal and SS RBCs become mature in vivo and may also be involved in the formation of dense dehydrated SS cells.

The inhibitory effect of internal Mg on K transport is not an exclusive property of SS cells, but is also observed in the least dense fraction of normal human RBCs.[4] A similar inhibitory action of Mg has been described by Lauf for the volume-dependent K flux of low K SRBCs.[12] Many of the properties of this K transport pathway in human RBCs are similar to those of the KCl cotransport described by Lauf in SRBCs.[7] Like human RBCs, high K sheep reticulocytes have a volume- and chloride-dependent pathway that is then inactivated in mature cells.[13] This pathway is present in all density fractions of low K SRBCs. Volume regulation through this pathway has been recently demonstrated in sheep reticulocytes.[14] In both human (SS and AA) and LK SRBCs, inhibition of K transport is produced not only by internal Mg, but also by other divalent cations, such as Ca and Mn. In both LK SRBCs and SS cells, Mn seems to be the most potent inhibitor, since complete inhibition of K efflux is achieved with internal Mn concentrations below 150 μmol/L.

Mg has also some important effects on other cation transport systems in RBCs: its presence is required for the activity of the Na-K ATPase,[15] of the Ca-ATPase,[16] and of the Ca-activated K channel.[17] External (but not internal) Mg has a marked inhibitory effect on the Na and K fluxes mediated by the bumetanide-sensitive Na-K-Cl cotransport.[18]

The Mg effect and other properties differentiate the K transport system we describe here from the deoxygenation-induced fluxes of SS cells. The volume-, pH-, and chloride-dependent K movement that we have described in carbonmonoxide treated SS cells (and in CC and AA cells) is inhibited by cell Mg, is insensitive to DIDS (4,4′-diisothiocyanostilbene-2,2′ disulforic acid), is not associated with an increased Na influx, and markedly decreases cell volume.[2-4] Preliminary reports also suggest that chloride-dependent K movement is inhibited when SS cells are deoxygenated.[19]

The deoxygenation-induced K movement in SS cells is chloride-independent[20] and partially inhibited by DIDS,[21] is associated with a marked increase of the Na permeability,[20,21] and does not produce changes in cell volume.[20] Deoxygenation of SS cells induces a marked increase of K and Na permeabilities and also of the internal free Mg concentration.[22] These findings seem to indicate that the deoxygenation-induced Na and K fluxes move through transport pathways different from the system we describe and are mediated instead by pathways that are influenced by membrane stretching.[23]

Mg is known to have important biologic regulatory functions.[24] In human RBCs, the total cell Mg content is 2 to 3 mmol/L/cell (3 to 4 mmol/kg/cell water) and the free Mg concentration is 0.3 to 0.4 mmol/kg/cell water.[10,25] ATP and other nucleotides are the most important RBC buffer system for Mg,[10] and only a small fraction of Mg is bound to cell proteins or 2,-3-DPG. Total RBC Mg content is elevated in the least dense fraction of normal RBCs and decreases as cell density increases.[26] Because the least dense fraction contains the youngest circulating RBCs, this observation could partially explain previous reports of elevated RBC Mg in sickle cell anemia and in other anemias characterized by marked reduction of RBC life span.[27,28] Preliminary reports on total Mg content of density-separated SS cells suggest that the total Mg content is lower in irreversibly sickled cells.[29] Clearly, future measurements of both, total and free RBC Mg contents, will have to be performed in SS erythrocytes separated according to density. The ratio of free to bound Mg in RBCs is markedly affected by the state of oxygenation of hemoglobin. Upon deoxygenation, ATP binds to deoxyhemoglobin, releasing Mg, and the free Mg concentration markedly increases.[22] One study reported that the K and Mg concentrations in normal erythrocytes (and lymphocytes) are positively correlated, so that cells with a higher Mg concentration tend to have a higher K concentration and therefore a higher water content.[30]

Genetic factors play an important role in determining the erythrocyte Mg content.[31] Because the Mg content is genetically determined, and since Mg has such an important effect on cell volume and K transport, it is possible that the genetic regulation of cell Mg is one of the "additional" genetic modulators of sickle cell disease.[32]

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Refinement of Lymphoma Cytogenetics by the Chromosome 18q21 Major Breakpoint Region

By Edward Lipford, John J. Wright, Walter Urba, J. Whang-Peng, Ilan R. Kirsch, Mark Raffeld, Jeffrey Cossman, Dan L. Longo, Ajay Bakhshi, and Stanley J. Korsmeyer

A small (2.8-kilobase, kb) major breakpoint region localized to segment 18q21 rearranges in $>$70% of t(14;18)(q32;q21) lymphomas. This rearrangement interrupts the *Bcl*-2 gene and introduces it into the Ig locus at 14q32. The rearrangement between the joining region (J_H) of Ig on chromosome 14 and the 18q21 region creates a translocation-specific DNA rearrangement. We generated probes that distinguish the 14;18 juncture on the derivative (der) 14 and der (18) chromosomes, providing a molecular approach to t(14;18) identification. Approximately 60% of unselected follicular lymphomas, 20% of diffuse large cell lymphomas, and 50% of adult undifferentiated non-Burkitt lymphomas demonstrated 14;18 rearrangements within the major breakpoint region. Examination of DNA for 14;18 rearrangements resolved the identity of 14q+ chromosomes in two patient's cells that lacked an obvious reciprocal partner. Identification of the exact restriction fragments that mediate translocations complements routine cytogenetics. The detection of DNA rearrangements does not require dividing cells or the presence of an identifiable reciprocal partner and can detect clonal translocation rearrangements when the neoplastic cells are only a minority of all cells present.

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SPECIFIC CHROMOSOMAL translocations occur in a wide variety of neoplasms, suggesting that these rearrangements may play a direct role in tumor pathogenesis. Moreover, particular types of neoplasms possess a unique or highly associated translocation.[1,2] These findings generated the thesis that genes could be identified flanking these breakpoints and would participate in transformation.[3] Human lymphoid neoplasms are proving a fruitful model to examine this postulate. The phenotypic landmark genes of Ig and the T cell receptors within B and T cell tumors, respectively, have proven to be the sites of interchromosomal recombinations.[4-6] Established c-*onc* genes or putative transforming genes have been identified at the far side of these breakpoints.[7-15]

We and other researchers previously cloned the chromosomal breakpoint on the der (14) chromosome of the t(14;18)(q32;q21) that is found in $\sim$80% of follicular B cell lymphomas.[10-12] Analysis of the der (14) revealed that a new gene, *Bcl*-2, from chromosome segment 18q21 moved into the immunoglobulin (Ig) locus at the 5′ end of joining (J_H) regions.[10-17] In contrast, we noted that diversity (D_H) regions of Ig mediated the juncture with 18q21 on der (18) chromosomes.[17] This argues that the t(14;18) occurs early in development at the first step of Ig gene rearrangement when a pre-B cell is attempting a D_H to J_H rearrangement.[17] We also noted that a small 2.8-kb major breakpoint region (mbr) on chromosome segment 18q21 was the site of rearrangement in $>$70% of the t(14;18) lymphomas.[17] Consequently, we prepared probes that identify the sites of 14;18 chromosomal juncture on the der (14), as well as the der (18) reciprocal partner. Because the t(14;18)(q32;q21) translocation is the most common translocation found in human lymphomas[18,19] and since a high percentage (70%) of these can be detected by gene rearrangement, we used this molecular marker of translocation to approach unresolved issues in lymphoma cytogenetics. Karyotypic analysis of lymphomas can sometimes result in an indeterminant classification of 14q+; in some series, this classification was $\sim$20% of cases.[20,21] This occurs because a der (14) chromosome is obvious but no certain reciprocal partner can be identified. Many of these 14q+ chromosomes appear to break at 14q32. In this study, we used 14;18 gene rearrangement to demonstrate that 14q+ lymphomas can be typical t(14;18) translocations.

MATERIALS AND METHODS

Isolation and analysis of DNA. High-mol-wt DNA was isolated directly from lymph node biopsies, from cells teased free from such biopsies, or from cell lines. Nuclei were first prepared by lysing cells in 0.25% Triton X-100, 0.25 mol/L sucrose, 50 mmol/L Tris (pH 7.5), 25 mmol/L KCl, and 5 mmol/L MgCl$_2$ on ice. Nuclei were subsequently washed twice in the sucrose buffer without Triton X-100 and were resuspended in this buffer and 0.3/mg/mL proteinase K, 1% sodium dodecyl sulfate (SDS), and 15 mmol/L EDTA (tetrasodium, pH 7.5) was added for 3 hours at 37°C. Samples were extracted with phenolchloroform until clean; the DNA was precipitated and samples were resuspended in 10 mmol/L Tris and 0.1 mmol/L EDTA. These genomic DNAs were digested to completion with *Hind*III or *Bam*HI restriction endonuclease, size-fractionated over agarose gels by electrophoresis, and transferred to nitrocellulose paper.[22] Purified cloned DNA restriction fragments were radiolabeled with ^{32}P by random hexanucleotide priming for use as probes.[23] Genomic blots were hybridized to ^{32}P probes with specific activities of 1 to 5 $\times$ 10^8 cpm/μg in 10% dextran sulfate, 40% or 50% formamide, 4$\times$ SSC, 1$\times$ Denhart's solution, and 10 μg/mL salmon sperm DNA. Blots were washed three times in 2$\times$ SSC, 0.1% SDS at room temperature, and twice in 0.1$\times$ SSC, 0.1% SDS at 50°C, and autoradiography was performed.

Preparation of probes. Genomic DNA probes from the mbr of chromosome segment 18q21 involved in the t(14;18)(q32;q21) translocation were constructed (Fig 1A). A 1.5-kb *Hind*III-*Eco*RI

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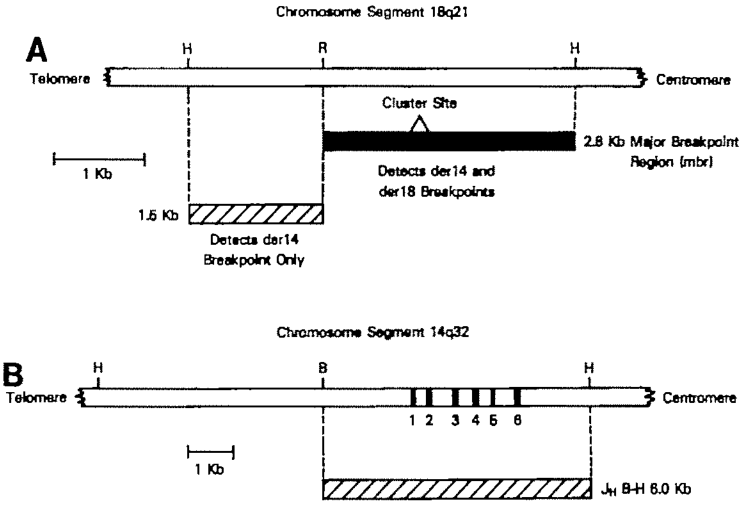


Fig 1. (A) Chromosomal orientation of the 4.3-kb *Hin*dIII genomic region of 18q21 where most t(14;18) breakpoints occur. These chromosomal breakpoints cluster within a small 150-bp region of the 2.8-kb *Eco*RI-*Hin*dIII major breakpoint region (mbr). When used as a probe, this 2.8-kb fragment recognizes the interchromosomal rearrangement on both the (der) 14 and (der) 18 breakpoints. In contrast, the telomeric 1.5-kb *Hin*dIII-*Eco*RI fragment detects the chromosomal breakpoint on only the (der) 14. (B) The 6.0-kb *Bam*HI-*Hin*dIII fragment containing the joining (J_H) segments used to detect rearrangements of the normal immunoglobulin heavy chain gene on chromosome 14 as well as the chromosomal breakpoint on the (der) 14.

fragment was initially isolated from the der (14) chromosomal breakpoint of cell line SU-DHL-6.[11] A region flanking this fragment was previously shown to be of chromosome 18 origin by somatic cell hybrids.[17] We determined the exact chromosomal location of this 1.5-kb *Hin*dIII–*Eco*RI fragment that would be used as a probe. This fragment was subcloned into pBR327, nick-translated with ^{3}HdNTPs and used in chromosome in situ hybridization as previously described.[4] Analysis of 71 metaphases from phytohemagglutinin (PHA)-stimulated lymphoblasts of a normal male revealed that 8.5% of all grains (14 of 165) were located on the long arm of chromosome 18 at band q21 (Fig 2). By Poisson analysis, the *Hin*dIII-*Eco*RI 1.5-kb probe was uniquely located at band 18q21 ($P \ll .05$). This probe was used to isolate a cosmid clone containing a 45-kb insert from the germline region of chromosome 18.[17] The entire 4.3-kb *Hin*dIII fragment containing the major site of rearrangement in t(14;18) lymphomas was subcloned from this cosmid isolate. A 2.8-kb *Eco*RI-*Hin*dIII fragment constitutes the centromeric portion and possesses the 150-base pair (bp) cluster site where most of these translocations focus (Fig 1A).[17] Consequently, this probe is capable of detecting the site of interchromosomal juncture on both the der (14) and der (18) reciprocal products in ~70% of t(14;18) translocations. In contrast, the 1.5-kb *Hin*dIII-*Eco*RI region lies telomeric to this breakpoint site and is translocated to the der (14) chromosome. Therefore, this 1.5-kb probe detects only the der (14) breakpoint rearrangement in *Hin*dIII-digested DNA. *Hin*dIII digests of lymphoma DNAs were comigrated on agarose gels with DNA from nontranslocated control sources of cells (fibroblasts, normal leukocytes), and Southern blots were hybridized with these two probes.

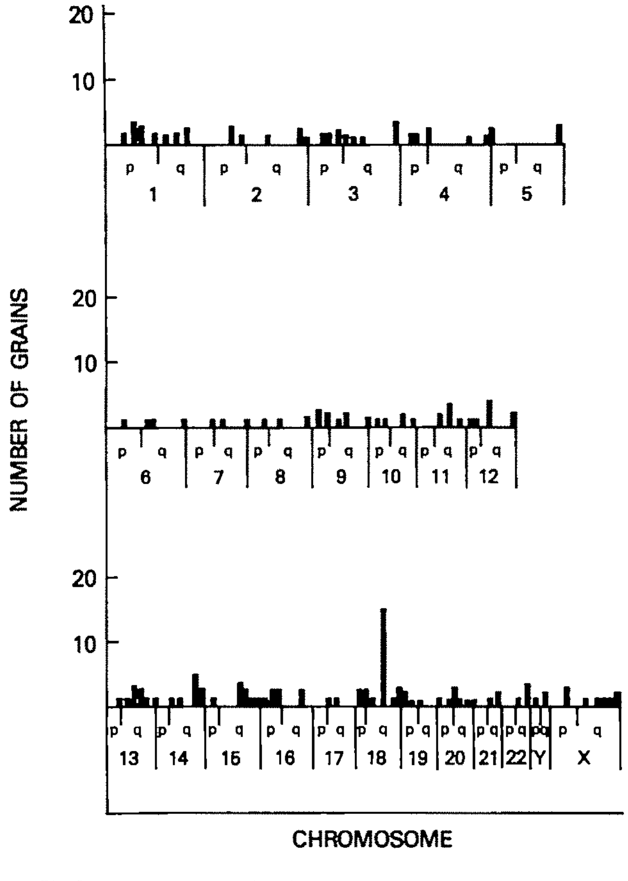


Fig 2. Bar graph of distribution of grains by chromosomal in situ. The *Hin*dIII-*Eco*RI 1.5-kb probe was localized to chromosome segment 18q21 with no other significant site of distribution.

The t(14;18)(q32;q21) breakpoints are also clustered on chromosome 14, occurring at the 5′ end of immunoglobulin joining (J_H) subsegments.[10-12,16,17] Therefore, we used a 6.0-kb *Bam*HI-*Hin*dIII probe containing the J_H region that recognized an 11.0-kb *Hin*dIII fragment in its germline form (Fig 1B).[24] This probe detects both the rearranged V/D/J allele residing on the normal chromosome 14 as well as the site of chromosomal juncture on the der (14) chromosome. These two J_H rearrangements can be discriminated by hybridizing the J_H probe and the chromosome 18 *Hin*dIII-*Eco*RI 1.5-kb probe to comigrated *Hin*dIII-digested lymphoma DNA. A single rearranged *Hin*dIII fragment containing the site of 14;18 juncture will hybridize to both probes (Fig 1).

RESULTS

A 14q+ lymphoma is a t(14;18) by rearrangement of the major breakpoint region. Lymphoma case 731, a diffuse large cell lymphoma, possessed a karyotype with a 14q+ chromosome but no obvious reciprocal partner in all 18 metaphases examined (Fig 3). Two normal chromosome 18s were noted, and no candidate for a der (18) was observed. Despite this, Southern blot analysis with the chromosome 18q21 origin *Hin*dIII-*Eco*RI 1.5-kb probe and the J_H probe from 14q32 demonstrated a 14;18 rearrangement (Fig 4). Comigration of *Hin*dIII-digested DNA from case 731

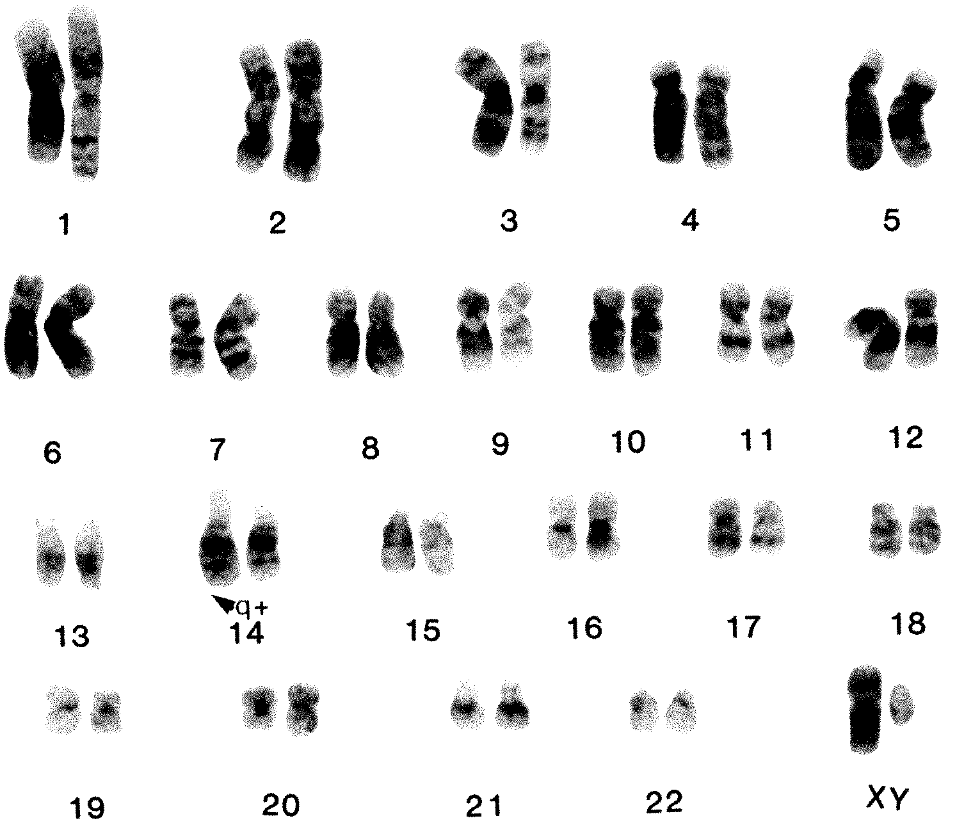
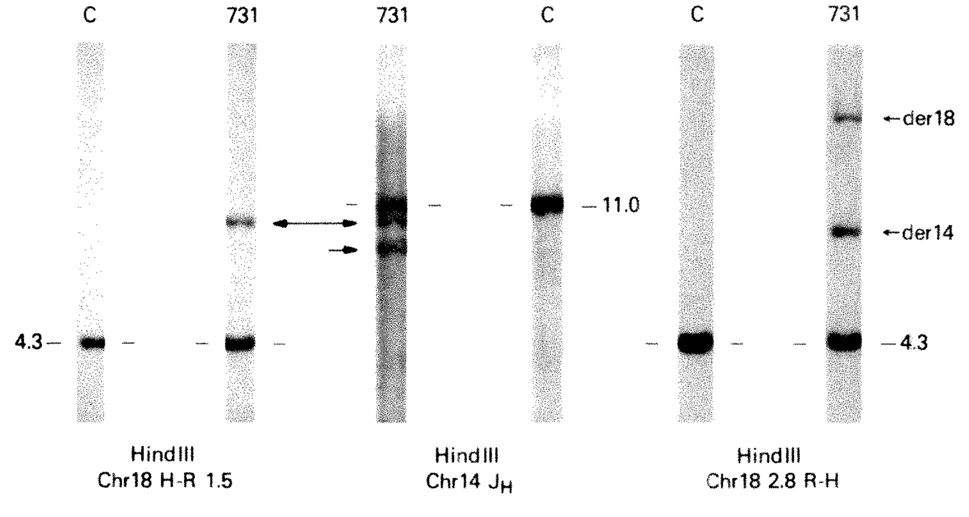


Fig 3. Cytogenetic analysis of lymphoma case 731 revealed a 14q+ chromosome with no obvious reciprocal partner.

revealed a single rearranged fragment that hybridized with both the chromosome 14 and 18 probes (Fig 4). This could also be demonstrated in *Bam*HI-digested DNA, in which a rearranged fragment possessed both J_H and chromosome 18 information (not shown). The 1.5-kb *Hind*III-*Eco*RI probe (Fig 1A) detected the site of 14;18 rearrangement on the der (14) in case 731 (Fig 4). We also used the 2.8-kb *Eco*RI-*Hind*III mbr as a probe (Fig 1A), and it detected sites of 14;18 rearrangement of both a der (14) and (18) chromosome (Fig 4). Although a der (18) chromosome was not seen in the available metaphases of case 731, molecular genetic examination proved that this reciprocal interchromosomal rearrangement was still present within some lymphoma cells.

Loss of the der (18) chromosome in a t(14;18) cell line. Another lymphoma provided the opportunity to observe the loss of a derivative chromosomal partner over time. A newly established cell line RL-7 was initiated from a diffuse undifferentiated (small noncleaved, non-Burkitt) lymphoma. Cytogenetic evaluation of the initial six metaphases after 30 days of culture indicated the presence of a 14q+ chromosome and two normal chromosome 18s without an obvious reciprocal partner (Fig 5). Southern blot analysis of this RL-7 cell line revealed a typical 14;18 der (14)

Fig 4. Genomic DNA from a lymph node biopsy of case 731 was digested with *Hind*III and comigrated on gels with a control (C) source of DNA with germline genes. Hybridization with the chromosome 18 *Hind*III-*Eco*RI 1.5-kb probe and the immunoglobulin J_H region reveals a common rearranged *Hind*III fragment identifying the (der) 14 chromosomal breakpoint (dual-headed arrow). The chromosome 18 *Eco*RI-*Hind*III 2.8-kb probe detects a (der) 18 as well as the (der) 14 breakpoint. Germline position of the 4.3-kb chromosome 18 region and the 11.0-kb immunoglobulin J_H region (--).



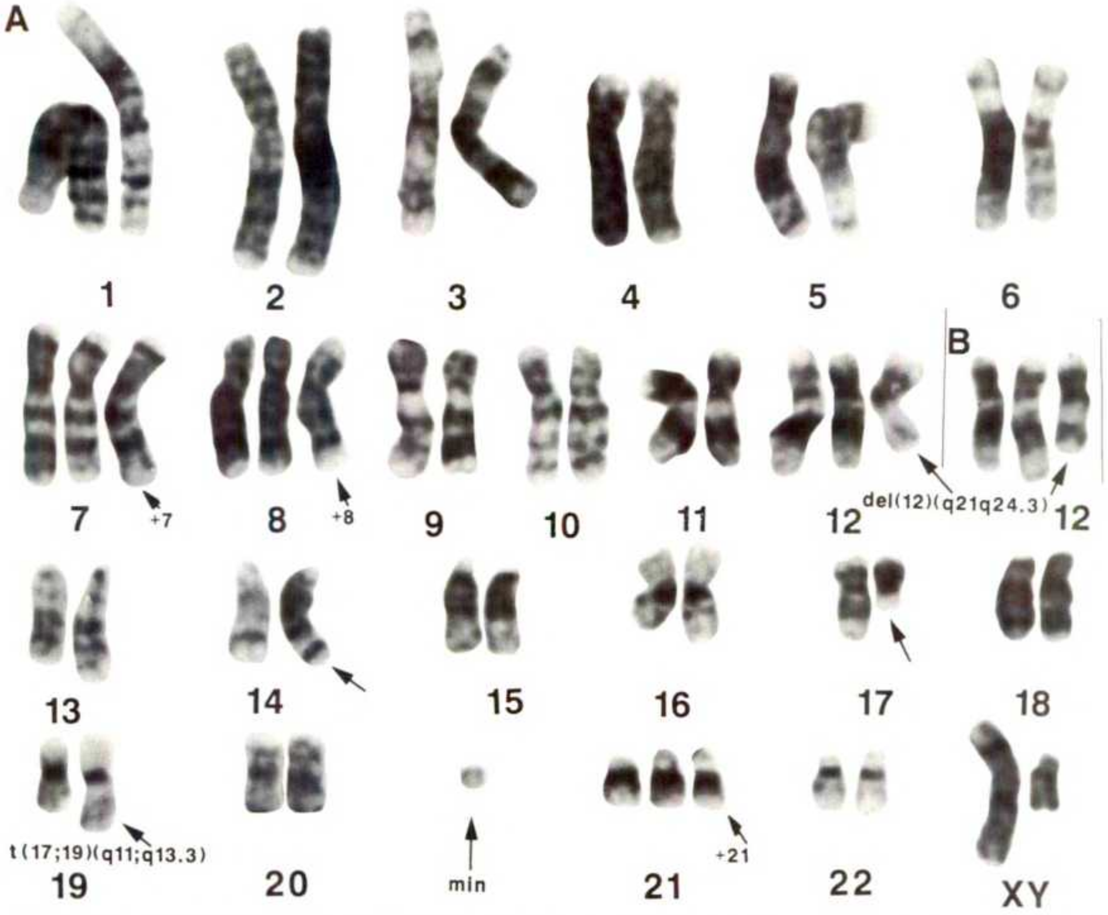


Fig 5. Cytogenetic analysis of lymphoma cell line RL-7 at day 30 revealed a 14q+ chromosome but no obvious reciprocal partner. Numerous other cytogenetic abnormalities were present, but two normal chromosome 18s were observed: A complete G-banded karyotype from cell A and a partial karyotype from cell B. Cell A had 51 chromosomes: 51, XY, +7, +8, +21, +del (12) (q21q24.3), 14q+, t(17;19) (q11; q13.3), +min. Cell B: partial karyotype showed normal 12 and the deleted 12.

chromosomal rearrangement (Fig 6). The identical rearrangement of the der (14) breakpoint and of the normal chromosome 14 Ig gene was present in the fresh lymphoma cells obtained from ascites as well as the cell line (Fig 6). Thus, the original lymphoma in vivo and the in vitro cell line were identical with respect to their t(14;18) translocation. Cytogenetic analysis of the original ascites lymphoma cells revealed the presence of a der (18) chromosome together with two normal chromosome 18s (Fig 7). Therefore, this lymphoma appeared to undergo a process of clonal evolution in which the normal chromosome 18 was duplicated and the der (18) was lost. This prompted a more extensive cytogenetic examination of metaphases from the RL-7 cell line at various time points after establishment. Table 1 shows that all 20 metaphases of the original in vivo lymphoma cells from ascites had a der (18) and two normal chromosome 18s. In contrast, the der (18) appears to be progressively lost over time in the RL-7 cell line. At day 1 and 2 of culture, all metaphases possessed a der (18) chromosome, whereas by 1 month <50% had a der (18) (Table 1).

Unaltered breakpoint rearrangement during histologic progression from follicular to diffuse lymphoma. Molecular analysis of the ascites cells (A 1431) and the RL-7 cell line revealed the presence of some der (18) type rearrangements (Fig 8), as would be predicted by the more extensive cytogenetic analysis. The identical der (14) as well as der (18) breakpoint rearrangements were present in the diffuse undifferentiated cells of the A1431 ascites and the RL-7 cell line. Moreover, lymph node biopsy (658) performed 5 years earlier exhibited a follicular (mixed small and large cell) histology yet also possessed the identical der (14) and der (18) rearrangements (Fig 8). Thus, the histologic progression from follicular to a diffuse undifferentiated lymphoma occurred in the same neoplasm. In addition, this progression to a more aggressive lymphoma was not associated with any further rearrangement of either chromosomal breakpoint (Fig 8). Lymph node biopsy 658 illustrates the sensitivity of the gene rearrangement approach in detecting clonal translocation rearrangements even when the neoplastic cells are a minority of all cells present (Fig 8).

18q21 major breakpoint region (mbr) rearrangements in nonfollicular lymphoma. The 18q21 major breakpoint region (mbr) rearranges and thus contains the chromosomal breakpoint in ~60% (11 of 17) of unselected follicular lymphomas we have examined in this and prior studies.[11] By extrapolation, this argues that >70% of follicular lympho-

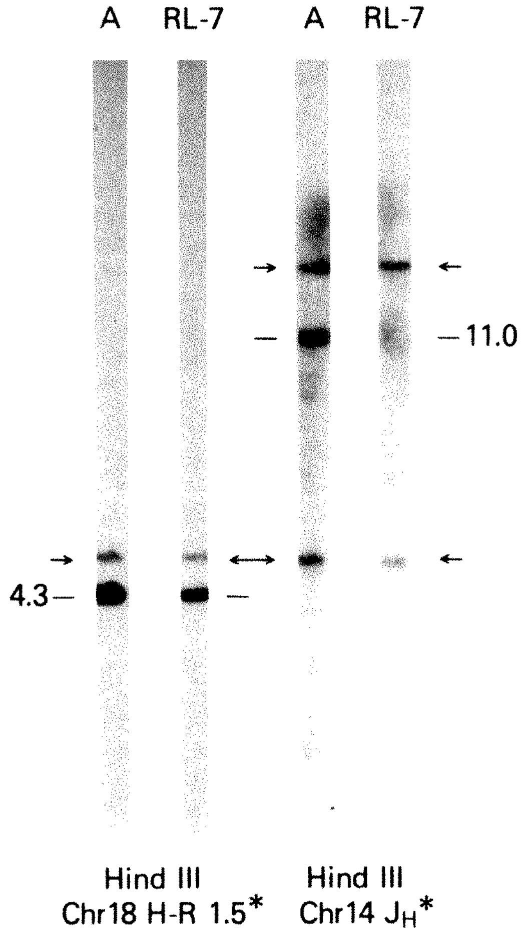


Fig 6. Genomic DNA from the original ascites cells of lymphoma case 1431 (A) was compared with the established cell line (RL-7). A single rearranged *HindIII* fragment hybridized to both the chromosome 18 and chromosome 14 probes and identified the 14;18 juncture on the (der) 14 (dual-headed arrow). Germline-sized *HindIII* restriction fragments (--). The larger sized rearrangement detected by the J_H probe (single-headed arrows) results from the V/D/J on the normal chromosome 14.

mas that actually possess a t(14;18) translocation by cytogenetics cluster their breakpoints within this region. Follicular lymphomas can progress over time to more aggressive neoplasms of diffuse large or undifferentiated cell types.[25] We wished to estimate the incidence of 18q21 mbr rearrangement within lymphomas not of follicular type. Within diffuse large cell lymphomas, 4 of 20 (20%) demonstrated a rearrangement within the mbr (Table 2). Adult undifferentiated (small, noncleaved) non-Burkitt lymphoma represents a histologic subset of diffuse aggressive lymphomas.[26] The features that are believed to distinguish these undifferentiated lymphomas from Burkitt lymphoma are an increased variation in the size of nuclei and the prominence of nucleoli. Adult undifferentiated non-Burkitt lymphomas are almost always of B cell type. They are usually diffuse, but nodular patterns have been noted, suggesting that they might be of follicular center cell origin.[27] Four of eight (50%) examined revealed a 14;18 rearrangement (Table 2). Only one of these cases represented a known histologic progression from prior follicular lymphoma (case 1431).

DISCUSSION

The presence of unique or highly associated chromosomal translocations in distinct types of neoplasms implies that their breakpoint regions are near genes contributing to neoplasia.[3] These characteristic translocations can often be discerned by routine cytogenetic analysis of metaphase spreads with the aid of banding techniques. The accuracy and efficiency of cytogenetics has improved considerably in recent years such that definitive chromosomal analysis is possible in most cases of non-Hodgkin's lymphoma. Even these techniques, however, are not uniformly successful in determining the precise nature of the interchromosomal partners, as the 14q+ lymphomas illustrated in this study. Instances of unresolved karyotypes often reflect the limitations inherent in cytogenetic techniques.

We used the well-circumscribed chromosomal breakpoint within the Ig J_H region on chromosome 14 and a defined 2.8-kb mbr on chromosome 18 to demonstrate t(14;18) translocations in lymphomas with unresolved cytogenetic assignments. Because most of these breakpoints cluster within a very small 150-bp region of 18q21, we were able to construct probes capable of discriminating the site of interchromosomal rearrangement on the der (14) *v* the der (18) chromosome (Fig 1). This capability provides the opportunity to follow the natural history of these two reciprocal partners throughout the course of lymphomas. In case 731, only 18 metaphases could be obtained; all of these demonstrated a 14q+, but no obvious reciprocal partner was present. Gene rearrangement studies proved this to be a juncture with 18q21 on the der (14), however, and demonstrated the presence of der (18) type rearrangements within the lymphoma biopsy tissue. This indicates that the der (18) chromosome was present only in a subpopulation of cells or that, alternatively, this juncture site was buried within an unrecognized complex translocation. The A1431 lymphoma and its cell line (RL-7) provided the opportunity to document an evolution of the chromosomal pattern over time. The initial uncertainty in cytogenetic classification of the RL-7 cell line resulted from a duplication of the normal chromosome 18 and the loss of the der (18). The t(14;18) results in the movement of the *Bcl*-2 gene from 18q21 to the der (14). Thus, the duplication of a normal chromosome 18 would result in a copy number of 3 for *Bcl*-2. Using a discriminatory S_1 protection assay, we determined, however, that only the translocated *Bcl*-2-Ig fusion product is transcribed whereas the *Bcl*-2 gene on the normal chromosome 18 is suppressed.[15] Perhaps this particular der (18) chromosome was mitotically unstable and simply prone to nondisjunctional loss. Prior examples of the duplication of normal chromosome 18, the presence of unpaired der (14)s, as well as duplication of the der (18) have been noted.[18-21] Both cases studied here illustrate that in many situations it may prove to be more efficient and conclusive to demonstrate the presence of a specific gene rearrangement than to be dependent on the availability of larger numbers of metaphases.

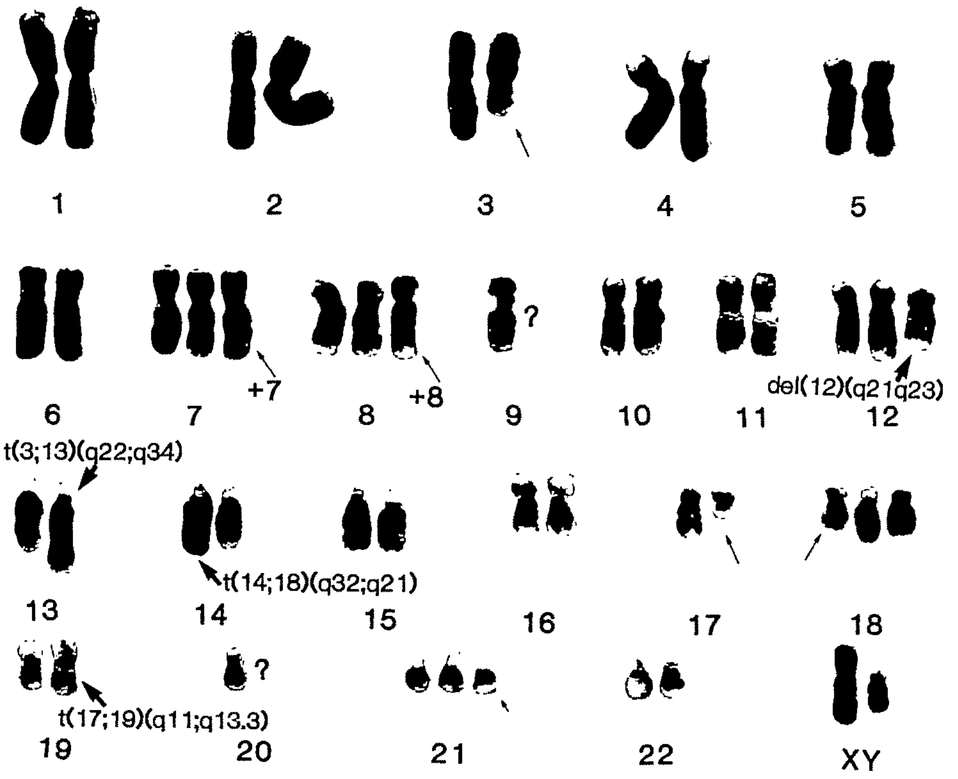


Fig 7. Cytogenetic analysis of fresh lymphoma ascites cells of case 1431 revealed the presence of a t(14;18)(q32;q21) with an 18q− as well as a 14q+ chromosome. In addition, two normal chromosome 18s were present.

The t(14;18) translocation is most highly associated with follicular lymphomas in which perhaps 85% of the small cleaved cell type bear the translocation.[18,19] Curiously, structural analysis of the t(14;18) breakpoint indicates that it occurs at a pre-B cell stage of development despite the mature B cell phenotype of the ultimate lymphoma. Moreover, follicular lymphomas followed over time may progress to diffuse large cell histology in up to 40% of instances.[28] We found typical 14;18 rearrangements in ~20% of the diffuse large cell lymphomas examined (Table 2). This is a lower incidence than in unselected follicular lymphomas (60%), suggesting that many diffuse large cell lymphomas do not arise from prior follicular center cell based lymphomas. Other histologic entities, including immunoblastic lymphoma (Table 2), chronic lymphocytic leukemia, pre-B cell acute lymphoblastic leukemia, and Burkitt lymphoma lacked 14;18 rearrangements.[11] In contrast, many of the adult undifferentiated non-Burkitt lymphomas demonstrated 18q21 mbr rearrangements, supporting previous speculation that this entity may often have a follicular center cell origin.

Table 1. Cytogenetic Characterization of Original Lymphoma and Cell Line

Source of Cells	Time (No. of Metaphases)	Chromosome 14 and 18 Findings
Ascites cells (No. 1431)	(20)	20: der (14), der (18), two normal 18s
RL-7 cell line	Day 1 (6)	5: der (14), der (18), two normal 18s 1: der (14), der (18), one normal 18
	Day 2 (2)	2: der (14), der (18), two normal 18s
	Day 5 (9)	8: der (14), der (18), two normal 18s 1: der (14), two normal 18s, no der (18)
	Day 30 (16)	7: der (14), der (18), two normal 18s 9: der (14), two normal 18s, no der (18)

Thus, use of a single probe from the mbr at 18q21 can detect the breakpoint rearrangement in >70% of t(14;18) follicular lymphomas as well as in some diffuse lymphomas. Southern blot detection of translocation rearrangements is capable of complementing and refining routine cytogenetics in several settings. Definitive DNA breakpoint rearrangements do not require the presence of an obvious reciprocal chromosomal partner, do not require the lymphoma cell to be dividing, and can detect translocated cells even if they comprise but 1% to 5% of all cells present. In addition, the remaining 30% of t(14;18) rearrangements will also be definable by using the cloned genomic regions that encompass and flank the 5′ and 3′ ends of the *Bcl*-2 locus.[29-31] Indeed, a restricted minor breakpoint region may exist at the 3′ end of this gene.[29] Molecular recognition of variant breakpoint sites will be considerably enhanced by use of additional technologies. This includes pulsed-field gel electrophoresis, in which it is possible to detect chromosomal rearrangements even if the breakpoint falls several hundred kilobases beyond the classic region.[32,33] Moreover, chromosomal in situ hybridization as shown in Fig 2 can be performed directly on tumor cells and demonstrate definitive

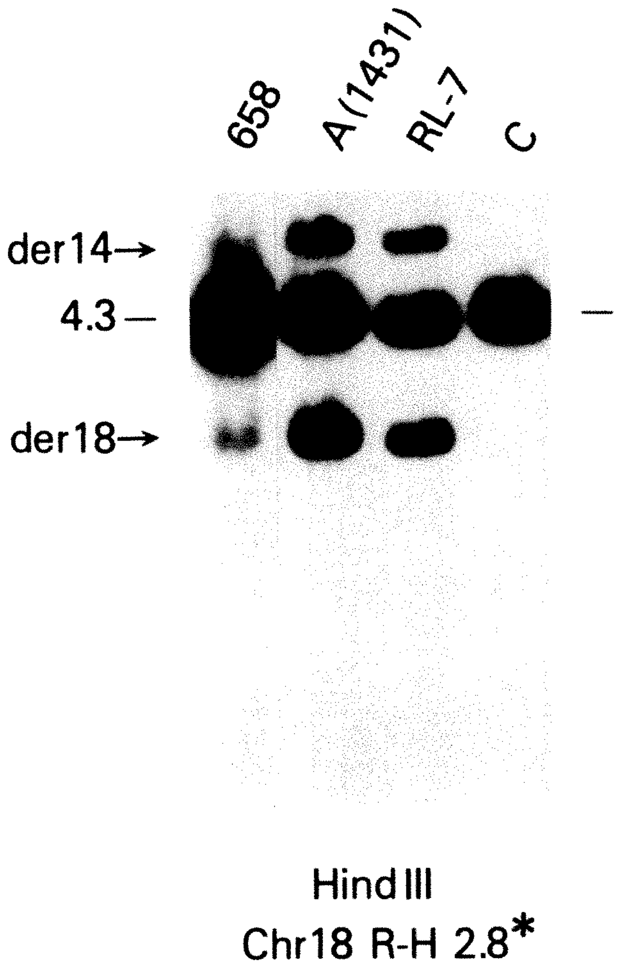


Fig 8. *EcoRI-HindIII* 2.8-kb major breakpoint region probe detects the presence of (der) 18 as well as (der) 14 chromosomal rearrangement sites (arrows). The 4.3-kb germline chromosome 18 region was present (--) on the normal allele and within admixed nonlymphoma cells. The identical (der) 14 and (der) 18 rearrangements were present in the diffuse undifferentiated cells of the ascites A(1431) and cell line (RL-7) as well as the preceding follicular lymphoma (658).

Table 2. Mbr Rearrangement in Nonfollicular Lymphoma

Case No.	Phenotype	18q21 mbr/J_H
Diffuse large cell lymphoma		
P 731	U	R
P 933	T	G
P1477	B	G
P1461	U	G
P1511	B	G
P1653	B	G
P1748	B	G
P1400	B	G
P1066	B	G
P1063	B	R
P1080	B	G
P1039	B	G
K1187	B	R
K1185	B	R
K1308	B	G
K158	H	G
K1186	B	G
K1217	B	G
K1218	B	G
K1219	B	G
Adult undifferentiated non-Burkitt lymphoma		
P397	T	G
P454	B	G
P592	B	G
P1310	B	R
P1312	B	R
P1431	B	R
P1530	U	R
P1541	B	G
Large cell immunoblastic lymphoma		
P1517	B	G
P1531	B	G
P1459	H	G
P1110	U	G
P1685	T	G
P1639	U	G

B, B cell; T, T cell; U, undetermined; R, rearranged; G, germline.

movement of a portion of one chromosome to a derivative chromosome. The molecular cloning of this and other clustered regions of chromosomal breakage should lead to refinements in the detection and definition of translocations.

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Influence of Hydroxyurea on Fetal Hemoglobin Production In Vitro

By Barbara A. Miller, Orah Platt, Sandra Hope, George Dover, and David G. Nathan

Cytotoxic drugs increase circulating fetal hemoglobin levels. We examined the mechanism by measuring the fetal hemoglobin produced per BFU-E–derived erythroblast following hydroxyurea treatment in vivo and in vitro. Treatment of four sickle cell patients increased the percentage of circulating F reticulocytes. The frequencies of bone marrow or peripheral blood BFU-E or CFU-E–derived colonies and their fetal hemoglobin content were unaffected. In all cases, the number of erythroid cells/progenitor-derived colony increased. To explore further the effect of hydroxyurea on fetal hemoglobin production, we added 50 µmol/L hydroxyurea to cultures of peripheral blood BFU-E–derived erythroblasts on 1 of 9 days (day 5 through 13) to nine samples. These BFU-E were derived from the peripheral blood of normal donors, sickle trait donors, and sickle cell anemia patients and from the bone marrows of monkeys. This concentration of hydroxyurea was selected so that the frequency of BFU-E and their size was moderately decreased. Addition of hydroxyurea to these progenitor-derived erythroid cells had no effect on fetal hemoglobin content per cell. Neither did transient exposure of progenitors to hydroxyurea prior to culture in nontoxic concentrations (0 to 500 µmol/L) result in a significant increase in fetal hemoglobin content in progenitor-derived erythroblasts. These data suggest that hydroxyurea does not directly alter the HbF program expressed by progenitor-derived erythroid cells. Instead, it enhances hemoglobin F content secondarily, possibly by inducing alterations in erythropoiesis.

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CELL CYCLE-dependent cytotoxic drugs recently have been shown to increase fetal hemoglobin (HbF) levels in non-human primates and in humans. When administered at appropriate doses and schedules, both S and M phase-specific agents have this capability.[1-7] Clinical studies of these drugs suggest that they may be useful in the treatment of certain patients with sickle cell anemia.[3,6] 5-Azacytidine (5-Aza) was the first S phase-specific agent to be studied.[2,3] The mechanism of its HbF-enhancing effect was believed to be related to its capacity to demethylate putative promotor regions of γ globin genes, which in turn stimulate γ globin expression.[2,3] Our experience with hydroxyurea indicated that hypomethylation immediately 5′ to γ genes may be a secondary effect of such drugs.[6] Other mechanisms of action have since been proposed: cytoreduction and rapid recruitment of erythroblasts directly from progenitors with higher fetal hemoglobin programs,[7-9] and direct induction of downstream maturation of progenitor cells.[8] Studies of baboons treated with 5-Aza and of monkeys with 5-Aza, hydroxyurea, and vinblastine support the cytoreduction hypothesis.[7,8] Clinical studies with 5-Aza and hydroxyurea suggest a complex action, due at least in part to a direct nontoxic effect on progenitors and precursors.[6,10]

We report measurements of BFU-E–derived erythroblast fetal hemoglobin content in four patients with sickle cell anemia (SCA) treated with hydroxyurea. In addition, hydroxyurea was added to cultures of peripheral blood BFU-E–derived erythroblasts to determine whether a direct effect on fetal hemoglobin production could be demonstrated. Two of these patients had previously responded to hydroxyurea in vivo.[6] No direct effect of hydroxyurea on fetal hemoglobin synthesis in BFU-E–derived erythroblasts was demonstrated in vitro, but treatment with hydroxyurea in vivo stimulated individual erythroid progenitors to form larger colonies of erythroid precursors in vitro.

These results demonstrate that hydroxyurea does not directly alter the HbF program established in erythroid progenitors or erythroid precursors derived from progenitors. It appears, instead, to exert its HbF stimulatory effect by its effect on erythropoiesis.

MATERIALS AND METHODS

Bone marrow and peripheral blood samples. Patients 1 through 4 had sickle cell disease with protracted painful crises and were treated, after informed consent, with a 3-day (patients 3, 4) or 5-day (patients 1, 2) course of hydroxyurea at 50 mg/kg/day divided into three oral doses. All patients had normal renal function as measured by serum creatinine, BUN, and creatinine clearance, but these measurements do not imply that the patients cleared the administered hydroxyurea at a rate similar to that of normal subjects. Results of treatment of patients 1 and 2 were previously reported.[6] Bone marrow and peripheral blood samples were drawn in preservative-free heparin or acid-citrate-dextrose (ACD) for assessment prior to treatment and 18 to 72 hours following completion of hydroxyurea therapy.

Peripheral blood samples were also obtained from normal AA volunteers, an AS parent, and from SCA patients aged >3 years who were not transfused within 6 months, and had no painful crisis within 7 days. Bone marrow samples were also obtained from two normal *M. fasicularis* monkeys who had not been bled or treated previously with any cytotoxic drugs.

Informed consent was obtained within institutional guidelines prior to treatment and procurement of blood and bone marrow samples. Simian marrow samples were obtained with the kind assistance of Dr Norman Letvin, under the guidance of the Commit-

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tee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.[11]

Analysis of percentage of F reticulocytes and percentage of HbF in peripheral blood. Analysis of percentage F reticulocytes in the peripheral blood of SCA patients was performed as previously described.[12] Analysis of percentage of HbF in the peripheral blood was performed by the alkali denaturation technique.

Methyl cellulose culture of peripheral blood and bone marrow mononuclear cells. Blood and marrow samples were diluted with tissue culture medium (Iscove's modified Dulbecco's medium, IMDM), layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged for 20 minutes at 2,200 rpm at 18°C. The cells at the interface were collected, washed, and plated at 1 to 2 × 10^5 cells/mL in 35-mm Petri dishes (Falcon) in a mixture containing 0.9% methylcellulose, 30% fetal calf serum (FCS, Gibco, Grand Island, NY), 9.0 mg/mL deionized bovine serum albumin (BSA, Sigma, St Louis, fraction V), 1.4 × 10^{-4} mol/L β-mercaptoethanol, 5% Mo cell line-conditioned medium as a source of BPA (generously provided by Dr David Golde) and with 2.0 U/mL crude erythropoietin (Toyobo) in IMDM. The plates were incubated in humidified 4% CO_2 at 37°C for 7 and 14 days.

The frequency of bone marrow CFU-E (7-day culture) and BFU-E (14-day culture) and of peripheral blood BFU-E–derived colonies/1 × 10^5 cells was assessed in patients who had been treated in vivo with hydroxyurea. The number of cells per erythroid colony, the picograms of fetal and total hemoglobin, and the percentage of fetal hemoglobin per progenitor-derived cell were also measured. Approximately 50 to 100 progenitor-derived colonies were plucked per sample, an aliquot was counted, and the average number of erythroid cells per colony was determined. When samples were plucked in duplicate or triplicate, the mean number of cells per colony ±1 SD was determined. Statistical significance of changes noted following treatment was determined with Student's paired *t* test.

Hydroxyurea (Squibb, Princeton, NJ, 97.6% pure) was added to erythroid culture dishes on days 3 through 13 following initiation of cultures derived from normal controls, AS controls, patients with SCA, and two monkeys. The drug was brought to a final concentration of 0 to 100 μmol/L, added at only one time point per dish, and was allowed to remain in the dish until completion of the 14 days of culture. In a second group of experiments, samples were pretreated with 5 to 500 μmol/L hydroxyurea in IMDM with 20% FCS and 2 U/mL erythropoietin (Amgen, Thousand Oaks, CA) at 37°C for 18 hours, washed, and then cultured. The number of BFU-E–derived colonies, the number of cells per colony, and the hemoglobin content of BFU-E–derived erythroblasts were assessed at 14 days. In some experiments, 5-Aza was substituted for hydroxyurea at a final concentration of 0.01 mmol/L.

Radioligand assay of HbA and HbF. Erythroid colonies were "plucked" and counted. A precisely measured volume was transferred to a second tube and pelleted, and the pellet was resuspended in 0.5 mg/mL carrier sheep Hb (300 to 400 μL) containing 0.02% Na-azide. This suspension was sonicated, microcentrifuged, and stored at 4°C.

Radioligand assay of total or fetal hemoglobin in picogram per BFU-E–derived cell was performed as previously described.[13,14] Each sample was assayed in triplicate to determine picograms of fetal or total hemoglobin. The percentage of HbF in such cells was calculated from the mean picograms of fetal and total hemoglobin measurements.

RESULTS

Effect of in vivo hydroxyurea treatment on in vitro hemoglobin F production. All four SCA patients treated with a single course of hydroxyurea immediately responded with increased circulating F reticulocytes (shown in Table 1). This represented an absolute increase since neither total RBCs nor percentage of total reticulocytes were affected. In general, F reticulocyte levels peaked at 7 days, remained elevated for at least 14 days, and returned to baseline by 21 days after initiation of treatment. A significant change in circulating HbF was not observed in this brief treatment period. To determine the effect of this treatment on the capacity of bone marrow BFU-E and CFU-E and peripheral blood BFU-E to proliferate and to produce fetal hemoglobin in the erythroblasts to which they give rise, BFU-E–derived colonies were cultured from the marrow and peripheral blood of patients prior to the initiation of hydroxyurea treatment

Table 1. Effect of Hydroxyurea Treatment on Hemoglobin F Production in BFU-E–Derived Cells

	Patient 1		Patient 2		Patient 3		Patient 4	
	Day 0	Day 7	Day 0	Day 8	Day 0	Day 4	Day 0	Day 4
F Reticulocytes (%)	16.0 ± 2.0	33.3 ± 1.2	8.7 ± 1.2	26.5 ± 5.6	25.5 ± 5.6	32.6 ± 6.7	4.0 ± 2.0	13.3 ± 4.2
Bone marrow								
Colonies*	ND	ND	146 ± 18.1	65 ± 8	141 ± 12	263	134	59
No. of cells/colony†	—	—	4.9 ± 0.3	8.0 ± 3.3	1.4 ± 0.2	2.1 ± 0.2	0.49	2.4 ± 0.2
HbF/cell (pg)‡	—	—	4.3 ± 1.3	4.1 ± 0.1	3.1 ± 0.1	3.6 ± 0.1	ND	2.1 ± 0.3
Total Hb/cell (pg)‡	—	—	—	16 ± 3.4	15.7	17.9 ± 0.6	17.1 ± 1.9	10.8 ± 0.2
HbF (%)‡	—	—	—	26.9	26.4	17.3	21.3	19.7
Peripheral blood								
Colonies*	271 ± 17	234 ± 5	67	—	127 ± 3.1	122	174	120
No. of cells/colony†	3.4 ± 0.5	4.8 ± 1.9	4.3 ± 0.4	12.3	5.6 ± 0.4	7.8 ± 0.6	2.4 ± 0.3	3.9 ± 0.4
HbF/cell (pg)‡	2.5 ± 0.3	2.3 ± 0.2	5.4 ± 0.1	4.82	4.2 ± 0.2	3.8 ± 0	3.0 ± 0.1	3.7 ± 0.2
Total Hb/cell (pg)‡	14.2 ± 1.2	13.6 ± 1.6	23.0 ± 0.7	19.2 ± 2.3	15.7 ± 2.3	14.9 ± 0.7	10.9 ± 0.2	11.9 ± 1.3
HbF (%)‡	17.8%	17.2%	23.7%	25.1%	26.7%	25.7%	27.4%	30.7%

Effect of a course of hydroxyurea at 50 mg/kg/day on hemoglobin F production in the peripheral blood and in BFU-E–derived erythroblasts of four SCA patients. Patients 1 and 2 were treated for 5 consecutive days, and patients 3 and 4 were treated for 3 days (day 1, first day of treatment).

*Number of BFU-E–derived colonies/1 × 10^5 bone marrow or peripheral blood mononuclear cells.

†Number of erythroid cells × 10^3/BFU-E–derived colony.

‡Picograms of hemoglobin F, total hemoglobin, and percentage of HbF in BFU-E–derived cells.

Table 2. Effect of Hydroxyurea on Hemoglobin F Production in CFU-E–Derived Cells

	Patient 2		Patient 3		Patient 4	
	Day 0	Day 8	Day 0	Day 4	Day 0	Day 4
F Reticulocytes (%)	8.7 ± 1.2	26.5 ± 5.3	25.5 ± 5.6	32.6 ± 6.7	4.0 ± 2.0	13.3 ± 4.2
CFU-E						
Colonies*	131 ± 27.0	248 ± 24	150	114	1,416 ± 183	1,376 ± 203
No. of cells/colony	285	418	260	520	148	273
HbF/cell (pg)	2.1 ± 0.2	2.0 ± 0.4	3.0 ± 0.1	2.9 ± 0.2	1.3 ± 0.4	1.2 ± 0.1
Total Hb/cell (pg)‡	9.2 ± 0.4	8.8 ± 0.3	11.1 ± 1.4	9.2 ± 0.8	5.1 ± 0.1	6.2 ± 0.9
HbF (%)‡	22.8%	22.7%	27.0%	31.8%	25.3%	18.5%

Effect of a course of hydroxyurea at 50 mg/kg/day on hemoglobin F production in the peripheral blood and in CFU-E–derived erythroblasts. Patient 2 was treated for 5 days, and patients 3 and 4 were treated for 3 days (day 1, first day of treatment).

*Number of CFU-E-derived colonies/1 × 10^5 bone marrow cells assessed in methyl cellulose (patients 2 and 3) or in plasma clot (patient 4).

†Picograms of hemoglobin F, total hemoglobin, and percentage HbF in CFU-E-derived cells assessed by radioligand assay.

and 18 to 72 hours following completion. Results are shown in Tables 1 and 2.

The effect of hydroxyurea treatment on the frequency of bone marrow and peripheral blood BFU-E–derived colonies 18 to 72 hours after cessation of treatment was variable, and statistically significant changes in colony number were not observed. In every case, however, the number of erythroid cells per BFU-E–derived colony increased following treatment (Table 1) and this change was statistically significant ($P < .05$). Despite the increase in the number of cells per BFU-E–derived colony and the wide range of HbF programs in the patients under study, the treatment failed to induce any change in fetal or total hemoglobin production per BFU-E–derived erythroid cell. Results of assessment of the influence of hydroxyurea treatment on proliferation and hemoglobin production in CFU-E–derived colonies are shown in Table 2. Again, no significant change in CFU-E–derived colony number was noted. CFU-E colony size was consistently larger after treatment, but results were not statistically significant ($P < .1$), possibly due to small sample size. No influence of treatment on the HbF program was expressed in progenitor-derived cells.

Influence of hydroxyurea on the hemoglobin F program in vitro. To assess the effect of hydroxyurea on the production of fetal hemoglobin by BFU-E–derived erythroblasts in vitro, 0, 1, 5, 25, 50, or 100 μmol/L hydroxyurea was added to culture dishes from SCA patients on day 7. Results of three experiments are shown in Table 3. Although colony number decreased with increasing hydroxyurea concentrations, no significant increase in fetal hemoglobin content was noted. The percentage of fetal hemoglobin decreased in two of these experiments following hydroxyurea addition, possibly because of a toxic effect since maximum fetal hemoglobin synthesis in culture occurs at this time period.[14] To determine whether the effect of hydroxyurea on hemoglobin synthesis is influenced by the stage of progenitor-derived cell maturation, the drug was added to peripheral blood BFU-E–derived cultures on days 3 through 13 of the culture period. It was added once to each dish and was not removed. A final concentration of 50 μmol/L was chosen because this dose reduced the number of BFU-E–derived colonies to 50% of control cultures when added on day 7, enough to demonstrate activity of hydroxyurea with a minimal cytotoxic effect. Addition of the drug at this concentration earlier than day 4 of culture resulted in significant inhibition of colony growth. Patients with a wide range of HbF programs were studied, including one AA adult, one AS parent, and five patients with SCA, three of them selected for their high HbF programs. Cultures of bone marrow BFU-E of two monkeys with elevated HbF programs were also studied. Results are shown in Fig 1. Despite addition of hydroxyurea on 9 different days of culture to nine samples with variable HbF

Table 3. Effect of Addition of Hydroxyurea to BFU-E–Derived Cells

	Concentration of Hydroxyurea (μmol/L)					
Experiment	0	1	5	25	50	100
Exp 1						
No. of colonies*	103 ± 14	ND	98 ± 4	48 ± 8	24 ± 2	2 ± 1
HbF (%)†	10.4	ND	9.1	12.7	10.3	ND
Exp 2						
No. of colonies	20 ± 4	24 ± 2	13 ± 1	10 ± 8	4 ± 1	1 ± 1
HbF (%)	25.2	23.8	16.5	14.5	ND	ND
Exp 3						
No. of colonies	26 ± 1	26 ± 0	18 ± 4	28 ± 1	20 ± 4	16 ± 4
HbF (%)	14.1	7.4	8.9	18.9	8.7	6.2

Effect of addition of 0 to 100 μmol/L hydroxyurea to day 7 BFU-E-derived colonies from the peripheral blood of three SCA patients.

*Number of BFU-E-derived colonies/1 × 10^5 peripheral blood mononuclear cells.

†Percentage of HbF in BFU-E-derived erythroblasts on day 14 calculated from measurement of picograms of total and fetal hemoglobin per cell by radioligand assay.

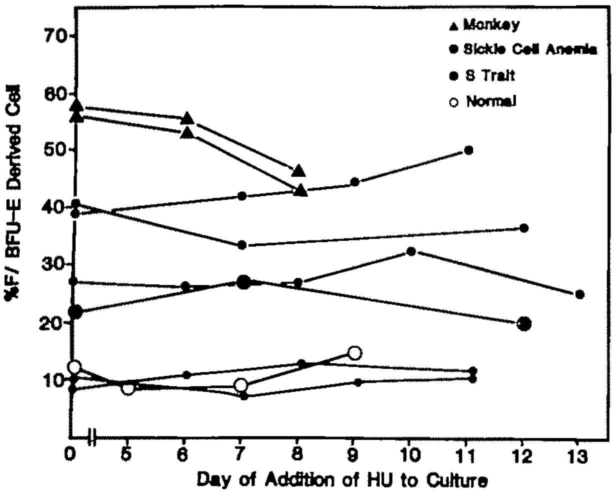

Fig 1. Influence of hydroxyurea on the HbF program in vitro. Percentage of hemoglobin F measured by radioligand assay in peripheral blood or bone marrow (monkey only) BFU-E–derived cells following addition of hydroxyurea at a final concentration of 50 μmol/L to methyl cellulose cultures on days 5 through 13. Day 0 indicates control cultures to which no hydroxyurea was added. Cultures were continued for 14 days, and assays were then performed.

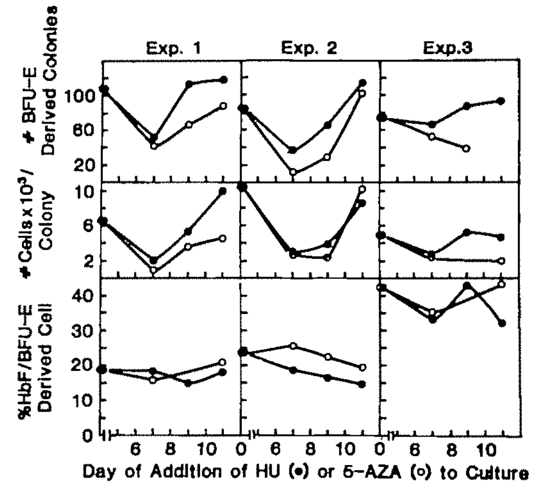

Fig 2. Influence of hydroxyurea and 5-Aza on peripheral blood BFU-E proliferation and fetal hemoglobin accumulation. Three patients were studied. The frequency of colonies (number/1 × 10^5 cells), the number of cells/BFU-E–derived colony, and percentage of fetal hemoglobin/BFU-E–derived cell were measured on day 14 following addition of 50 μmol/L hydroxyurea or 0.01 mmol/L 5-Aza to the cultures on days 4, 7, 9, or 11. Day 0 indicates control cultures to which no hydroxyurea was added. Shown are effects of the drugs on proliferation when added before day 12 of colony growth and lack of effect on HbF production.

programs, no significant effect of the drug on hemoglobin F production was detected.

The effect of a transient exposure to hydroxyurea was studied by pretreating progenitor cells with 0, 5, 50, 100, and 500 μmol/L hydroxyurea for 18 hours prior to culture. Results of three experiments are shown in Table 4. No change in colony number was observed following this short exposure despite the use of tenfold higher hydroxyurea concentrations than those used in continuous exposure experiments. No statistically significant increase in fetal hemoglobin content was observed, although small increases in fetal hemoglobin content were noted in some of the treatment groups.

Comparative influence of hydroxyurea and 5-Aza on peripheral blood BFU-E proliferation and hemoglobin synthesis. To compare the effect of hydroxyurea with that of 5-Aza on the BFU-E–derived erythroblast hemoglobin programs, three patients were studied in more detail. Hydroxyurea 50 μmol/L or 5-Aza 0.01 mmol/L was added to

Table 4. Pretreatment of BFU-E With Hydroxyurea

	Hydroxyurea (μmol/L)				
Experiment	0	5	50	100	500
Exp 1					
No. of colonies*	61 ± 4	52 ± 4	28 ± 4	25 ± 10	50 ± 8
HbF/cell (pg)†	3.6 ± 0.3	2.3 ± 0.2	4.0 ± 0.4	2.8 ± 0.3	4.6 ± 0.4
Total Hb/cell (pg)†	33.5 ± 0.9	15.4 ± 1.0	37.3 ± 0.5	24.9 ± 1.1	40.7 ± 0.1
HbF (%)	10.7	14.9	10.7	11.2	11.3
Exp 2					
No. of colonies	16.3 ± 3	18 ± 3	ND	23 ± 16	18 ± 2
HbF/cell (pg)	2.3 ± 0.2	1.8 ± 0.2	ND	3.1 ± 0.1	2.4 ± 0.2
Total Hb/cell (pg)	21.5 ± 1.1	17.1 ± 0.7	ND	19.9 ± 0.4	21.0 ± 1.4
HbF (%)	10.7	10.5		15.6	11.4
Exp 3					
No. of colonies	14 ± 1	18 ± 8	12 ± 1	14 ± 7	10 ± 3
HbF/cell (pg)	0.8 ± 0.1	0.7 ± 0.1	2.0 ± 0.2	3.1 ± 0.1	ND
Total Hb/cell (pg)	14.9 ± 2.3	19.9 ± 0.1	23.6 ± 3.0	33.6 ± 1.2	ND
HbF (%)	5.4	3.5	8.5	9.2	

Effect on hemoglobin F production by BFU-E–derived cells of pretreatment of peripheral blood mononuclear cells from three patients with SCA with 0 to 500 μmol/L hydroxyurea.

*Number of BFU-E–derived colonies/1 × 10^5 peripheral blood mononuclear cells.

†Percentage HbF in BFU-E–derived erythroblasts at day 14 calculated from measurement of picograms of total and fetal hemoglobin per cell by radioligand assay.

cultures of the peripheral blood BFU-E of three SCA patients on days 4, 7, 9, or 11. The influences of these treatments on the number of BFU-E/1 × 10^5 cells, number of cells per BFU-E–derived colony, and the hemoglobin program expressed as percentage of fetal hemoglobin per BFU-E–derived cell were assessed; the results are shown in Fig 2. When added prior to or on day 4, colony growth was nearly obliterated and the effect on fetal hemoglobin synthesis could not be determined. The number of BFU-E–derived colonies and the number of cells per BFU-E–derived colony were decreased following addition of either cytotoxic drug to the cultures, demonstrating that both drugs were active. Despite this dramatic effect on progenitor proliferation, neither 5-Aza nor hydroxyurea influenced the percentage of fetal hemoglobin produced in the progenitor-derived erythroid cells. Neither drug significantly altered the picograms of fetal hemoglobin per cell in treated colonies, even in cultures with decreased cell proliferation (data not shown).

DISCUSSION

The mechanism by which cell cycle-specific cytotoxic drugs stimulate HbF and F cell production are not known. Two broad hypotheses have been explored. One is based on drug-induced modifications of DNA (such as hypomethylation of globin promoter regions)[2,3] and the other on cytokinetic considerations.[7,9] The two may be related. For example, we previously determined that changes in DNA methylation can occur as a result of administration of hydroxyurea as well as 5-Aza.[6] Hypomethylation and F cell production may both be secondary manifestations of drug-induced differentiation.

To study this complex problem, we evaluated the effects of in vivo treatment with hydroxyurea on BFU-E and CFU-E–derived cells of four patients. Although the frequencies of CFU-E or BFU-E–derived colonies were not regularly affected, the proliferative capacities of these colony-forming cells were markedly enhanced without alteration in the quantity of HbF produced per progenitor-derived erythroid cell (the HbF program). Different results might have been obtained if additional points had been chosen for the study, but ethical considerations demanded that bone marrow aspirates be performed only twice. Because F reticulocytes remained elevated for 7 to 14 days after the posttreatment progenitor proliferation and HbF programs were assessed, we conclude that the sustained increase in F reticulocytes was not the result of a direct effect of the drug on the capacity of CFU-E or BFU-E–derived proerythroblasts to produce HbF. Rather, the data suggest that the drug influenced the number of proerythroblasts (and hence reticulocytes) produced by each progenitor.

We also assayed the percentage of fetal hemoglobin produced following treatment of maturing BFU-E–derived erythroblasts with hydroxyurea or 5-Aza in vitro. The ability of the radioligand assay of progenitor-derived cells to detect different HbF programs is clearly shown in Fig 1. The culture and assay system measures the accumulation of hemoglobins F and A following a 14-day period during which erythroblasts are derived from progenitors and mature. Drug treatment of BFU-E–derived erythroblasts as they mature in the culture permits analysis of the effects of treatment on erythroid cells that are nearly synchronized in a particular stage of maturation. This sharply contrasts with the treatment of bone marrow aspirates that contain cells of all stages of erythroid maturation. In addition, previous studies showed that this system has the capacity to detect factors that influence the quantity of fetal and adult hemoglobin produced per cell.[15,16] Neonatal progenitor-derived erythroblasts alter the percentage of hemoglobin F made in vitro following coculture with sodium butyrate[15] and γ-interferon (γ-IFN).[16] Adult progenitor-derived erythroblasts have been shown to produce less fetal hemoglobin following coculture with γ-IFN[16] and more fetal hemoglobin following bromodeoxyuridine treatment.[17] The data from the present study demonstrate that HbF production in progenitor-derived cells is not altered by treatment with hydroxyurea. Unlike other factors,[15-17] hydroxyurea cannot influence hemoglobin F production in erythroblasts directly derived from BFU-E.

The present results do not support the simple cytotoxic hypothesis that we[5-7] and other researchers[8] have previously presented. We suggested that the drugs kill mature and rapidly dividing erythroblasts and progenitors, forcing their replacement with erythroblasts immediately derived from spared progenitors.[18] Such progenitor-derived erythroblasts produce more fetal hemoglobin than do erythroblast-derived erythroblasts.[19] That concept was largely based on studies in simians in which the F cell response is regularly preceded by transient reticulocytopenia and loss of mature marrow erythroid progenitors (CFU-E).[5-7] Neither was observed in these human studies. Indeed, Dover et al recently observed that when hydroxyurea treatment resulted in marrow toxicity, hemoglobin production was *less* likely to increase.[20] SCA patients with minimal renal dysfunction exhibited the most cytotoxicity and the least F reticulocyte response to hydroxyurea.[20]

Our studies support a complex cytokinetic explanation for hydroxyurea-induced stimulation of the F reticulocyte response.[8] Most erythroblasts present in normal marrow are not directly derived from progenitors but are instead the products of previous erythroblast division.[18] Normal marrow erythroblasts contain 20 pg hemoglobin/cell but <1 pg to undetectable quantities of HbF per cell, whereas erythroblasts derived from normal peripheral blood or bone marrow BFU-E and CFU-E contain 20 pg total hemoglobin, and 1 to 2 pg fetal hemoglobin.[14] A brief course of hydroxyurea in vivo may result in stimulation of the proliferative thrust of surviving progenitors and/or promote their premature differentiation into erythroblasts.[21] Either event would increase the marrow fraction of progenitor-derived erythroblasts, which produce greater quantities of γ globin than do erythroblasts derived from erythroblasts. The extent to which the HbF per F cell rises in vivo is likely to depend on the inherent ability of the particular progenitor population to produce hemoglobin F.

In summary, the studies we report demonstrate that hemoglobin F production in progenitor-derived erythroblasts from AA, AS, and SCA individuals and monkeys is not directly altered in vitro by treatment with hydroxyurea. Treatment of SCA patients with hydroxyurea in vivo affects the numbers of progeny per erythroid progenitor but does not have any demonstrable influence on fetal hemoglobin production per progenitor-derived erythroblast. Because hydroxyurea does not appear to reregulate fetal hemoglobin production in differentiating erythroid cells, we propose that hydroxyurea secondarily enhances the synthesis of HbF by inducing alterations in erythropoiesis.

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δ Glycophorin (Glycophorin B) Gene Deletion in Two Individuals Homozygous for the S−s−U− Blood Group Phenotype

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Blood cells from two unrelated individuals whose erythrocytes exhibit respectively N S−s−U− and MN S−s−U− blood group phenotypes were examined by immunoblotting, periodic acid-Schiff (PAS) staining, and Southern blotting. Protein bands characteristic of δ glycophorin (glycophorin B) were absent from the immunoblots of whole erythrocyte lysates when probed with polyclonal glycophorin antisera and from isolated erythrocyte membranes stained with PAS reagents. Genomic DNA from the two individuals' leukocytes was digested with a panel of restriction enzymes and probed with α M glycophorin cDNA obtained from human K562 leukemic cell line. The *EcoRI, PstI,* and *KpnI* restriction site patterns were identical to those of S+s+U+ controls in fragment numbers and relative size but differed from controls in band intensities. Restriction mapping with *HindIII, PvuII, SacI, MspI,* and *BamHI* revealed that S−s−U− individuals lack fragments that are reproducibly observed in S+s+U+ controls, and most likely encode δ glycophorin. Using truncated 5′ and 3′ cDNA segments as probes and comparing, in control individuals, hybridization intensities of fragments with amino acid sequence homologies, we have inferred the assignment of restriction fragments to the α and δ glycophorin genes. Our results suggest that the absence of δ glycophorin in the two S−s−U− individuals is a result of deletion of the entire δ glycophorin gene. This is the first report of a glycophorin gene deletion.

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GLYCOPHORINS α and δ (glycophorins A and B) are glycoproteins of the human erythrocyte membrane that carry the antigens of MNSs blood groups.[1,2] Serologic family studies have established that αM and αN, as well as δS and δs glycophorins, are products of respective allelic genes.[3] Chemical structural analyses identified amino acid substitutions that distinguish αM from αN glycophorins and those that specify δS and δs glycophorins.[4-6] The U blood group phenotype is serologically closely associated with the S and s determinants, but the structural association of the U antigen with δ glycophorin is less clear.[7,8]

Glycophorins αN and δ are highly homologous, being identical within the amino terminal 26 residues and sharing homologies in their remaining sequences*.[1,5,6,9] Human α and δ glycophorin genes are closely linked on the long arm of chromosome 4.[3,6,10] Recently, evidence obtained from αM glycophorin cDNA and oligonucleotide probes indicated that α and δ glycophorins are encoded by separate and distinct single copy genes.[11,12]

Although a number of variant MNSs blood group phenotypes have been identified by serologic studies,[1,2,6] few have been characterized by protein and carbohydrate structural analyses.[13-20] To understand the genetic basis for the origin and expression of variant glycophorins, we have undertaken studies on the organization of the glycophorin genes in individuals exhibiting variant MNSs phenotypes. In this report we present evidence derived from protein and genomic DNA analyses that erythrocytes of two unrelated individuals homozygous for the S−s−U− blood group phenotype lack δ glycophorin as a result of a δ glycophorin gene deletion.

MATERIALS AND METHODS

Blood samples. Blood samples from two unrelated S−s−U− donors (M.E.K. and M.L.A.) and certain controls (S+s+U+) were provided by the Community Blood Center, Dayton, OH. Control blood samples were also obtained from laboratory personnel. The MNSsU blood group phenotypes of the variant erythrocytes were: donor M.E.K., M−N+S−s−U−; donor M.L.A., M+N+S−s−U−. Serologic typing was performed using sera available at the Consultation Laboratory of Gamma Biologicals. M and N status was tested with antisera from human, animal, and lectin sources. Anti-S, anti-s, and anti-U were human source reagents, and at least three samples of each were used. U-negative status was confirmed by absorption-elution studies.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses. Fresh erythrocytes were washed three times with phosphate-buffered saline (PBS) containing inhibitors of proteolysis and lysed by mixing 0.025 mL packed erythrocytes with 0.75 mL 1× (sodium dodecyl sulfate (SDS)-polyacrylamide gel) sample buffer.[21,22] Ten microliters of the whole lysate was subjected to electrophoresis on SDS-10% polyacrylamide gels.[21] Gels were blotted onto nitrocellulose as described by Towbin et al,[23] and glycophorins were visualized by autoradiography after consecutive incubations with one of two polyclonal rabbit antisera and then with ^{125}I-labeled protein A.[24] One antiserum was raised against intact α MN glycophorin, the second against the carboxyl terminal peptide of α MN glycophorin (peptide C, residues 82 through 131).[20] Whereas the glycophorin antiserum reacts with both α and δ glycophorins, the antipeptide C serum detects only α glycophorin and shows no crossreaction with δS or δs glycophorin.[20,21]

Erythrocyte membranes were prepared as previously described.[4] To 0.50 mL membrane suspension was added 0.25 mL 3× sample buffer and 10 μL subjected to electrophoresis. Gels were stained with Coomassie blue or periodic acid-Schiff (PAS) reagent.

Genomic DNA restriction mapping analysis. Genomic DNA was isolated from the peripheral leukocytes of EDTA-anticoagulated blood as described by Kunkel et al[25] with minor modifications. All DNAs were larger than 50 kilobases (kb) using intact λ DNA as marker.

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*Complete amino acid sequences of α and δ glycophorins are shown in Fig 1 of ref 12.

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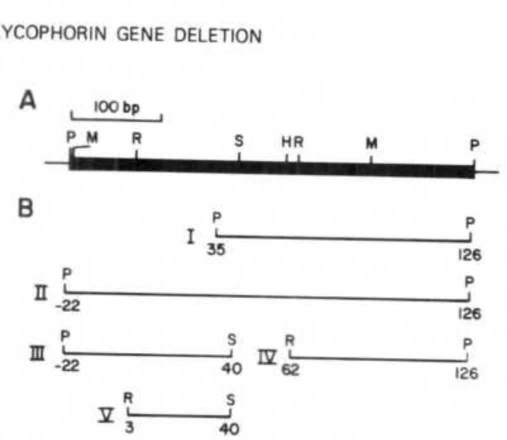

Fig 1. (A) Partial restriction map of α glycophorin cDNA insert. Restriction endonucleases are denoted as P, *Pst*I; M, *Mbo*I; R, *Rsa*I; H, *Hpa*II; and S, *Sac*I. (B) Probes used for nucleic acid hybridization. Roman numerals designate probes. Probes I and II were generated by *Pst*I from glycophorin cDNAs, pHGpA-1, and pHGpA-3, respectively[12]; probes III through V were generated, as shown, by cleavage of pHGpA-3 with *Pst*I, *Rsa*I, and *Sac*I. Numbers at beginning and end of each probe indicate amino acid residues of α glycophorin they encompass. cDNAs lack the codons of the last five residues of α glycophorin, which contains a total of 131 residues.

Genomic DNAs were digested with restriction endonucleases (New England BioLab, Beverly, MA; Boehringer Mannheim, Indianapolis), under conditions recommended by the manufacturers, in a ratio of 4 to 5 U enzyme/μg DNA at 37°C for 15 hours. Blotting transfer of DNA fractionated by agarose gel electrophoresis was performed as previously described.[26]

Recombinant plasmids, pHGpA-1, and pHGpA-3 harboring the glycophorin cDNAs, were isolated from transformed *Escherichia coli* RR1 cells[12] and further purified by cesium chloride density-gradient centrifugation as described.[27] A partial restriction map of α M glycophorin cDNA inserts was predicted from their nucleotide sequences (Fig 1A). The cDNA hybridization probes are depicted in Fig 1B (I through V). The probes were removed from the plasmids by appropriate restriction enzymes, purified by electrophoresis in low-melting agarose (Bio-Rad, Richmond, CA) or 6% to 8% polyacrylamide gels, excised and labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) using random primers (Pharmacia, Piscataway, NJ) and Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD) as described by Feinberg and Vogelstein.[28] The specific activities of these primer-extended probes ranged from 2.1×10^9 to 3.4×10^9 cpm/μg.

Nucleic acid hybridizations were performed essentially as described.[29] The hybridization and washing techniques were standardized as follows: To each blotted nitrocellulose filter (~200 cm^2) was added 10 mL hybridization solution containing 5 × SSC (1 × SSC = 0.15 mol/L NaCl + 0.015 mol/L Na$_3$ Citrate, pH 7.0), 5 × Denhardt (1 × Denhardt = 0.1% Ficoll, 0.1% polyvinylpyrrolidone (PVP) and 0.1% bovine serum albumin, BSA), 10 mmol/L EDTA (pH 8.0) and 200 μg/mL sonicated salmon sperm DNA. Prehybridization was carried out at 65°C for ~10 hours; hybridization was carried out overnight at the same temperature, following addition to the hybridization solution of the appropriate radioactive probe at a final concentration of 4 to 5×10^6 cpm/ml. Highly stringent washing conditions were as follows: 2 × SSC, 0.1% SDS at room temperature for 30 minutes and then at 60°C for 30 minutes, and finally in 0.1 × SSC, 0.1% SDS at 60°C for 1 hour. Occasionally, repeated hybridizations of a single nitrocellulose filter were carried out with different probes; in those cases, the bound probes were removed from the filter after each use by washing in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0) at 68°C for 1 to 2 hours. The blots were exposed to Kodak XAR-5 film against two intensifying screens at −80°C for 3 to 4 days.

RESULTS

Absence of δ glycophorin in S−s−U− erythrocyte membranes. Immunoblots show that the erythrocyte membranes of both S−s−U− individuals lack the δ glycophorin monomer and dimer bands (3b and 6) when compared with a normal control (Fig 2A). These bands are readily identified in the S+s+U+ control erythrocytes by reaction with antiglycophorin serum and lack of reaction with antipeptide C serum (Fig 2A and B).[21] The absence of δ glycophorin characteristically located in the PAS 3 band (2) is also seen in membrane preparations stained with PAS reagent (Fig 2C).

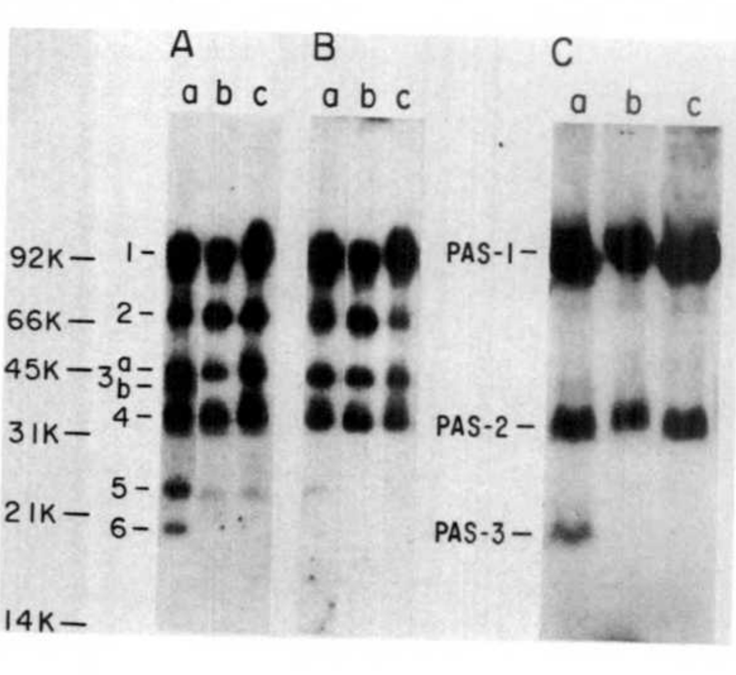

Fig 2. Glycophorins in erythrocytes and isolated membranes of S+s+U+ control and S−s−U− variants. (A and B) Immunoblots of SDS 10% polyacrylamide gels of intact erythrocytes probed with antiserum to α glycophorin (in A) and antiserum to α glycophorin carboxyl terminal peptide C (residues 82 through 131) (in B). (C) Schiff-stained gel of isolated membranes: (a) control M+ N+ S+s+U+ individual; (b) donor M.E.K. (M−N+ S−s−U−); (c) donor M.L.A. (M+ N+ S−s−U−). Vertical numbers (left) refer to mol wt markers and glycophorin bands present in erythrocytes of the control individuals; the pattern of the control individual's erythrocytes is representative of >100 individuals examined.[21] Bands 1 and 4 as well as PAS1 and PAS2 correspond to α glycophorin dimer and monomer; bands 3b and 6 correspond to δ glycophorin dimer and monomer respectively; PAS3 correspond to δ glycophorin.[2]

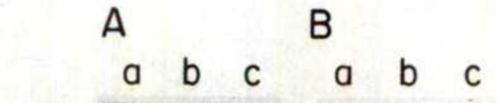

Fig 3. Autoradiograms of Southern blots of *EcoRI* (A) and *PstI* (B) digests of genomic DNA of S+s+U+ controls (a) and S−s−U− individuals [(b) donor M.E.K., (c) donor M.L.A.]; 7.5 μg DNA was resolved by 0.8% agarose gel electrophoresis, blotted, transferred, and hybridized with probe II (Fig 1B). Size markers for the restriction fragments (in kb) were the *HindIII* digests of λ DNA.

Genomic DNA restriction analysis. Genomic DNA of S+s+U+ control and S−s−U− individuals was first examined using restriction enzyme digestions and the α glycophorin cDNA insert from pHGpA-3 (probe II in Fig 1B) as hybridization probe. For all enzymes used except *Msp*I whose digests exhibit a restriction fragment length polymorphism (RFLP)[30] (described below), restriction site patterns shown in control are representative of DNA of ∼20 M, N, S+,s+,U+ individuals examined. For all these enzymes, the possibility that differences in restriction site patterns in DNA of S−s−U− individuals might result from RFLP is therefore excluded.

As shown in Fig 3, fragments of S−s−U− DNA digested with *Eco*RI and *Pst*I are identical to those of the DNA of control individuals except for the weaker intensity of the smallest size bands; this was also observed in control and variant DNA patterns with Kpn I digests (gels not shown). In contrast, *Hind*III, *Pvu*II, *Sac*I, *Msp*I, and *Bam*III digestions all demonstrated distinct restriction maps that showed the absence of specific bands in the DNA of S−s−U− individuals as compared with S+s+U+ controls (Figs 4 and 5). To identify fragments that encompass the amino (5′) and carboxyl (3′) terminal regions of α and δ glycophorins, pHGpA-1 and -3 cDNAs as well as three truncated probes generated from them were used. The sizes of the respective fragments are listed in Table 1; hybridization patterns are shown in Figs 4 and 5.

The restriction site patterns revealed by *Hind*III digestion and probe II hybridization show the absence in DNA of two bands 6.7 kb and 3.4 kb, in the S−s−U− individuals as compared with the control. From hybridization patterns of S+s+U+ DNA with the 5′ and 3′ specific probes, we conclude that these fragments may derive from δ glycophorin genes. (Table 1 and Fig 4A III and IV). The remaining *Hind*III fragments seen in the DNA of both S+s+U+ and S−s−U− individuals most likely derive from the α glycophorin gene.

The hybridization signals of the α glycophorin gene fragments are nearly equivalent, whereas those of bands absent in S−s−U− DNA differ significantly; under identical hybridization and washing stringency conditions, the intensity of the 3′ 3.4-kb fragment was consistently weaker than the intensity of the 6.7-kb amino terminus specific band (Fig 4A III and IV). These intensity differences further support the assignment of the hybridizing bands, absent in S−s−U− variants, to the δ glycophorin gene, because amino acid sequence homologies of α and δ glycophorins are much more extensive in the amino terminal than in the carboxyl terminal region of the glycophorin polypeptides*.[5,9,31] The remaining more strongly hybridizing bands found in both control and variant DNAs are correspondingly assigned to the α glycophorin gene. These observations support the conclusion that α and δ glycophorins are encoded by separate single-copy genes.[12]

Analysis of *Pvu*II hybridization patterns led to a similar conclusion (Fig 4B III and IV and Table 1). The 7.3-kb fragment of α glycophorin hybridizes strongly with the 3′ probes but weakly with the 5′ probes. Because the latter probes encode the α glycophorin polypeptide through residue 40 (Fig 1B), this suggests that an intron may be present in the vicinity of residues 35 through 40 and that the 7.3-kb and 1.9-kb fragments may contain all the coding sequences for α glycophorin.

*Sac*I digestion shows the absence in S−s−U− individuals of a single 8.0-kb fragment (Fig 4C and Table 1). In the control, this fragment hybridized with all probes used, and as noted previously in *Hind*III and *Pvu*II digests, 5′ probes III and V showed a much stronger hybridization signal than the carboxyl terminus-specific probe IV (Fig 4C). These observations strongly suggest that the 8.0-kb fragment contains the total coding sequence for δ glycophorin and may be large enough to include most of the noncoding sequences that immediately flank the δ glycophorin gene. The presence of a single *Sac*I fragment indicates, in addition, that the *Sac*I site residing within the α glycophorin coding region (Fig 1A) is eliminated in the δ glycophorin gene. This is in agreement with the amino acid sequence data that suggests a deletion in δ glycophorin of codons 27 through 55 of α glycophorin*.[9]

Restriction analysis using *Bam*HI and *Msp*I revealed even larger size DNA deletions in the two S−s−U− individuals (Fig 5A and Table 1). The *Msp*I 14.2-kb fragment includes

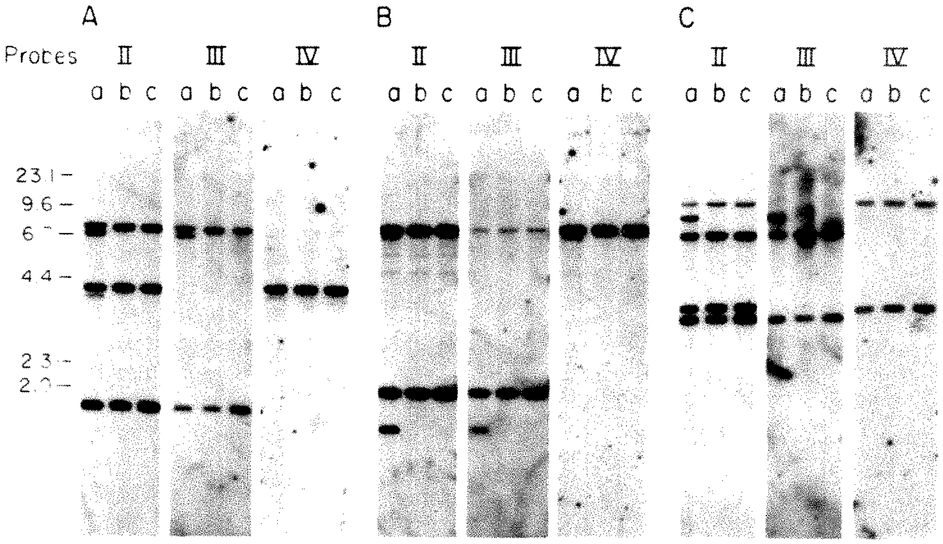

Fig 4. Autoradiograms of Southern blots of *Hind*III (A), *Pvu*II (B) and *Sac*I (C) digests of genomic DNA of S+s+U+ controls (a) and S−s−U− individuals [(b) donor M.E.K., (c) donor M.L.A.]. DNA 7.5 μg was resolved on 0.8% agarose gels and hybridized with probes shown in Fig 1B. When blots were rehybridized with probe V, a pattern identical to that observed with probe III was obtained. Size markers as in Fig 3.

the δ glycophorin gene, but the 11.7-kb fragment is one of a pair of RFLP alleles (the other one being the 7.3-kb fragment) linked to the α N glycophorin gene.[30] The 14.2-kb fragment is analogous to the *Sac*I 8.0-kb fragment in that it hybridized strongly with probe III but weakly with probe IV (Table 1). The 14.2-kb *Msp*I fragment most likely encompasses the total coding sequence of δ glycophorin gene. This is because the single cutting site for *Msp*I, within the coding region of α glycophorin gene

$$\left(\text{codons 58 through 59 or } \begin{matrix}\text{ACC} & \text{GGA}\\ \text{Thr} & \text{Gly}\end{matrix}\right)$$

may be absent in δ glycophorin gene due to a different codon specifying δ glycophorin at position 29. This residue in δ glycophorin is the position of the δS/δs allelic substitution, Thr and Met in δs and δS, respectively,[5] and is aligned with Thr 58 (ACC), in α glycophorin*.[9,31] Assuming a single base change between the codons, it is likely that the codon for Thr-29 in δ glycophorin would be ACG, given the unique codon for Met, ATG. Such an ACG codon would indeed eliminate the *Msp*I site. This conclusion is supported by our failure to differentiate SS, ss or Ss genotypes using *Msp*I.[30]

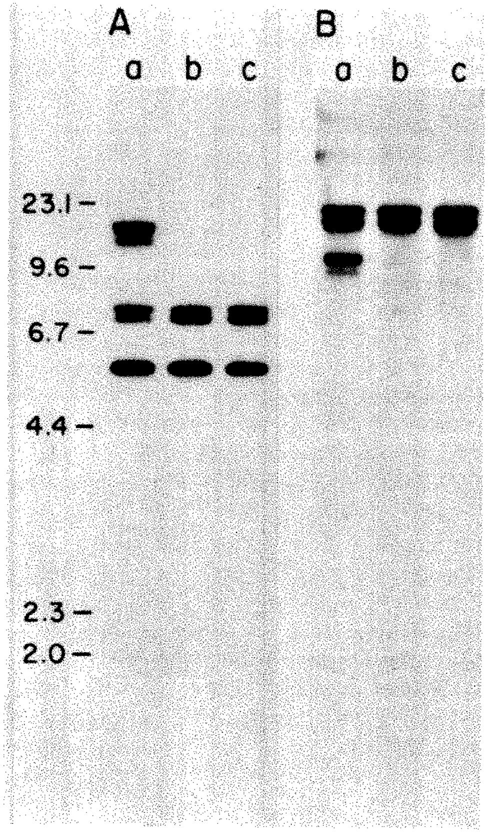

Fig 5. Autoradiograms of Southern blots of *Msp*I (A) and *Bam*HI (B) digests of genomic DNA of S+s+U+ control (a) and S−s−U− individuals: [(b) donor M.E.K., (c) donor M.L.A.]. DNA 7.5 μg resolved on 0.8% agarose gel and hybridized with probe II (Fig 1B). Size markers as in Fig 3.

DISCUSSION

In this report, we provide evidence that two unrelated individuals who exhibit the S−s−U− blood group phenotype lack erythrocyte δ glycophorin and that the absence of this protein correlates with deletion of the δ glycophorin gene. This is the first documentation of a glycophorin gene deletion. This was concluded primarily from comparative restriction mapping of genomic DNA from control and variant donors using as probes previously cloned cDNAs and probes derived from different regions of these DNAs that encode defined amino and carboxyl terminal regions of α and δ glycophorins. Specific fragments were absent in five different enzyme digests of S−s−U− DNA, that in control DNA hybridized with the 5′ or 3′ terminal probes or with both. The conclusion that the fragments missing in the variant individuals derived from δ glycophorin gene was inferred primarily from our ability to relate, in controls, the relative intensities of bands in given digests to regions of high and low homology in the amino acid sequences of α and δ

Table 1. Genomic DNA Restriction Fragments of S+s+U+ Control and S−s−U− Individuals: Hybridization of Restriction Fragments with cDNA Probes

		I* (35-126)		II (−22-126)		III and V^2* (−22-40) (3-40)		IV (62-126)	
Restriction Enzymes	Size (kb)	C†	V†	C	V	C	V	C	V
*Hind*III	7.4			+	+	+	+		
	6.7			+	−	+	−		
	3.7	+	+	+	+			+	+
	3.4	+	−	+	−			+	−
	2.5‡	+	+	+	+				
	1.8			+	+	+	+		
*Pvu*II	7.3	+	+	+	+	+	+	+	+
	6.7	+	−	+	−			+	−
	1.9			+	+	+	+		
	1.6			+	−	+	−		
*Sac*I	9.6	+	+	+	+			+	+
	8.0	+	−	+	−	+	−	+	−
	6.4			+	+	+	+		
	3.1	+	+	+	+			+	+
	2.7			+	+	+	+		
*Msp*I§	14.2			+	−	+	−	+	−
	11.7			+	−	+	−		
	7.8	ND§		+	+	+	+		
	7.3			+	+	+	+		
	5.8			+	+			+	+

Probes described in legend to Fig. 1B.

*Data observed with probes I and V are listed here but not shown in Fig 4. Numbers in brackets indicate amino acid sequence that probe encompasses.

†C, S+s+U+ control, V, S−s−U− variant; (+) indicates presence and (−) indicates absence of corresponding fragment.

‡Fragment shows low intensity of hybridization with probes I and II; its hybridization with the other probes was not detected.

§Restriction fragments for *Msp*I digests hybridized with probes III and IV not shown; hybridizations with probes I and V were not carried out (ND).

glycophorins. This allowed us to assign fragments as originating from the α or the δ glycophorin gene.

This study complements previous findings[12] and results in a more conclusive identification of the gene origin of restriction fragments. This became possible not only by comparison of control and variant maps, but also through the use of cDNA fragment probes. In contrast to previously used synthetic oligonucleotides that were complementary to α glycophorin cDNA sequences, the larger truncated probes could detect fragments derived from δ glycophorin genes despite possible mismatches (that might prevent oligonucleotide hybridizations) arising from sequence divergence of α and δ glycophorin genes.

Our conclusion that the entire coding sequence of the δ glycophorin gene is deleted is based primarily on the absence in S−s−U− donors of an 8.0-kb *Sac*I and a 14.2-kb *Msp*I fragment, fragments that were present in the S+s+U+ controls and hybridized with both the 5′ and 3′ hybridization probes; additional evidence was provided by the absence in the other digests of one pair of fragments, one hybridizing in control DNA strongly with the 5′ probe and the other with much less intensity with the 3′ probe. Although the sizes of the deleted fragments suggest that the entire δ glycophorin gene is deleted, using the cDNA probes we cannot now define the total size or the 5′ and 3′ endpoints of the deletion.

It became clear in the course of these studies that the restriction fragments observed in S−s−U− individuals originated exclusively from α glycophorin. The patterns shown by these fragments and their assignment to the regions of α glycophorin they encode are similar to those noted previously.[12] In addition to these observations, however, the use of the 5′ and 3′ terminus-specific cDNA probes revealed in several digests two fragments that originate from the amino terminus of α glycophorin gene and in the *Sac*I digests two carboxy terminus-specific fragments. Our data indicate at least two exons in δ glycophorin gene and suggest that the genomic organization of α glycophorin gene may be more complex.

The S−s−U− phenotype of erythrocytes of the two individuals examined here correlates with the lack of δ glycophorin in membranes of these cells; the demonstration that the δ glycophorin gene is deleted from both chromosomes in each individual provides proof that these phenotypes correlate with the genotypes of the donors: donor M.E.K., αN δ^{0}/αN δ^{0}; and donor M.L.A., αM δ^{0}/αN δ^{0}. Such a straightforward relationship may not necessarily be observed in all cases. Other mechanisms, including defects in transcriptional, translational, or posttranslational processing may be operative in other individuals of the S−s−U− phenotype.

The possibility of alternative origins for the phenotype was recently revealed through a study of a Dantu blood group variant.[32] As shown, erythrocytes of an individual exhibiting MN, Dantu, s+ phenotype lacked δ glycophorin, despite the s+ phenotype. We have explained this apparent inconsistency by demonstrating that the Dantu variant glycophorin is a hybrid δ-α structure, which evidently retains the s specific antigenic determinant in the δ glycophorin portion of the molecule. It will be of interest to determine if the δ glycophorin gene is deleted in Dantu individuals. The complexity of the glycophorin gene described here and further implied by our previous studies as well as by the presence of many serologically identified blood group variants, suggests that the glycophorin gene locus will be particularly informative in studies of human gene recombination.

Gene deletion is a common mechanism underlying some human genetic disorders, most notably thalassemias.[33] Certain thalassemias occur in geographically defined groups of individuals, for which it has been hypothesized that the absence of α or β globin gene may have represented a selective advantage. It is therefore of interest that the S−s−U− blood group phenotype is prevalent among selected populations, in particular among Blacks from certain regions in Africa where the incidence of this phenotype is 1% to 35%[34] as compared with an incidence of <0.001% in the white population. Our current studies are thus an important first step in understanding of the origin of the S−s−U− phenotype. It remains to be shown whether this phenotype is always characterized by the δ glycophorin gene deletion and whether when the deletion occurs a single origin or multiple evolutionary origins are implicated.

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Kinetic Evaluation of the Pool Sizes and Proliferative Response of Neutrophils in Bacterially Challenged Aging Mice

By Gerald Rothstein, Robert D. Christensen, and Buddy Ray Nielsen

Clinical observations during infection suggest that in aged patients, the kinetic or proliferative responses of neutrophils to infection may be deranged. To test this hypothesis, the neutrophil responses of 6-month-old and 30-month-old mice were compared. After intrapulmonary injection of *Escherichia coli*, young mice exhibited neutrophilia and diminution of the neutrophil storage pool (NSP) by a mean of 6.4×10^6 neutrophils/two femurs. This was accompanied by an increase in the pool of CFU-GM from a control value of 1.1×10^5 cells/two femurs (range 0.7 to 1.4) to 1.5×10^5 (1.1 to 1.9) ($P < .05$) and the thymidine suicide (relative proliferative rate) of CFU-GM rose from 27% (19 to 42) to 51% (31 to 61) ($P < .05$). Furthermore, the CFU-GM of infected young mice displayed enhanced differentiation to the neutrophil series. In contrast, old mice exhibited a greater mean diminution of the NSP: 12.8×10^6 neutrophils. Also, old mice experienced a reduction in CFU-GM from 2.3×10^5 (1.0 to 3.9) (controls) to 1.3×10^5 (1.2 to 1.5)/two femurs ($P < .05$), a reduction in the proliferation of CFU-GM and reduced differentiation of CFU-GM to neutrophils. These experiments establish that the neutrophil response of infected old mice is disordered, with exaggerated depletion of the NSP and lack of stimulus-driven granulocytopoiesis as reflected by a paradoxical reduction in the number and proliferative rate of precursors. This defect may be compounded by decreased differentiation of precursors to neutrophils.

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TO SUSTAIN an adequate neutrophil supply, an appropriate balance must exist between the kinetics of neutrophil release from the marrow, intravascular turnover of neutrophils, and the production of neutrophils by progenitors. An example of the changes that occur in this balance can be found during infection. In dogs infected with staphylococci, release of cells from the neutrophil storage pool (NSP) is accelerated acutely, resulting in an increase in the size of the blood neutrophil pool.[1] In rats infected with streptococci, outflow of neutrophils from the NSP is sufficient to diminish the NSP appreciably.[2] In compensation, the number and proliferative rate of progenitor cells increase and the marrow neutrophil reserve is restored.[2] Thus, the neutrophil response to significant infection includes of a series of events: release of neutrophils from the marrow, an elevation of the blood neutrophil concentration, and the activation of homeostatic mechanisms by which the number and proliferation of neutrophil precursors are increased to restore the neutrophil supply.

Clinical observations suggest that in aged humans, the neutrophil response to bacterial challenge may be deranged. Murphy and Fine reported a high incidence of neutropenia in aged patients with bacteremic pneumococcal pneumonia and correlated neutropenia with an increased risk for mortality.[3] Although not kinetically defined, these observations could reflect either disordered neutrophil release from the NSP, an increased marginal pool of blood neutrophils, or a diminished compensatory proliferative response. Such defects would be of clinical relevance, and the mechanisms responsible for them would be of basic biologic interest as well.

For these reasons, we compared the response of young and old C57Bl/6 mice to a standardized challenge with *Escherichia coli* K1. The effect of bacterial challenge on the sizes of the various pools of neutrophils was evaluated, as was the ability of the animals to respond by accelerating neutrophil production.

MATERIALS AND METHODS

Animals. Six-month-old or 30-month-old male C57Bl/6 mice were obtained from Charles River Laboratories (Stone Ridge, NY) under the auspices of the National Institute of Aging. Each of the animals was shipped in an individual one-mouse compartment and was housed individually at the University of Utah Vivarium until use within 1 to 6 weeks of arrival. When killed, animals were examined by autopsy for gross evidence of tumors, renal disease, hepatic disease, or inflammatory lesions. Animals exhibiting these abnormalities were excluded from the study.

Examination of the blood and bone marrow. Mice were narcotized by CO_2 inhalation, and blood was obtained from the vena cava. The nucleated cells were counted electronically, and differential counts of 100 cells were performed on Wright's stained smears. Absolute concentrations of neutrophils were calculated from the nucleated cell counts and differentials.

Bone marrow was flushed from both femurs according to the method of Chervenick and colleagues.[4] Cells were counted electronically and 500-cell differential counts were performed, using Wright's stained smears and established criteria for identification of cells.[4] Absolute numbers of the various marrow cells were calculated from the nucleated cell counts and differentials. All studies of hemic cells involved killing the animal; consequently, no serial measurements were performed in individual mice.

Cultures of CFU-GM. Cultures of CFU-GM were performed in 35-mm plastic dishes, using semisolid methylcellulose medium in a manner previously described.[5] Cultures contained 5% postendotoxin serum, a concentration that exerted maximal stimulatory effect for the formation of colonies from both young and old mice. Cultures were incubated for 7 days at 37°C in a 7% CO_2 atmosphere. Colonies (>50 cells) were scored with the aid of a stereomicroscope.

Thymidine suicide experiments were carried out in a manner similar to that described by Byron.[6] Marrow cells were incubated at 37°C for 20 minutes in 2 ml of Hank's Balanced Salt Solution (HBSS) containing 0.1 mCi ^{3}HTdR (New England Nuclear, North

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Billerica, MA) with a specific activity 80 Ci/mmol. Corresponding control cells were incubated in an equivalent amount of ^{1}HTdR. ^{3}HTdR uptake was inhibited competitively by adding 30 mL cold HBSS with 15% fetal calf serum (FCS) and 100 g/mL ^{1}HTdR. The cells were centrifuged at 200 × g for 12 minutes and were washed twice in the HBSS-cold thymidine serum solution. The thymidine suicide was taken as the proportional decrement in colonies when CFU-GM were pretreated with ^{3}HTdR, as compared with ^{1}HTdR.

The morphology of colonies was examined by aspirating all clearly isolated colonies from culture dishes with a capillary pipette, and then smearing them on coverslips for Wright's staining. Differential counts were performed on at least 50 cells for each colony; if sufficient numbers of cells were present, 100 cells were counted.

E coli expressing the K1 antigen were kindly supplied by Dr Harry Hill, University of Utah. Organisms were grown overnight in Tryptic Soy Broth (TSB) and washed three times in phosphate-buffered saline (PBS) prior to resuspension at 10^4 to 10^6 CFU/μL in PBS for injection with a Hamilton syringe. Concentrations of *E coli* were estimated by measuring the absorbance of suspensions at 600 nm, and the numbers of viable CFU were verified by quantitative culture in TSB agar.

Data were evaluated statistically with Student's *t* test, the Wilcoxon rank-sum text, or the Fisher exact test. Considering the usual sample sizes of four to eight values, these maneuvers are effective in evaluating the validity of apparent differences but could fail to detect more subtle differences that might not achieve statistical significance with relatively small numbers of values.

RESULTS

Weights of the 53 young mice (33.34 ± 0.32 g) (mean ± SEM) and the 50 old mice (33.29 ± 0.42 g) did not differ.

Mortality after inoculation with E Coli. Six-month-old (young) animals were inoculated transthoracically into the right lung with 1 μL/g body weight of a PBS suspension containing various doses of *E coli*. All five animals that received 10^6 *E coli*/g died within 48 hours. Of ten mice that received 10^5/g, six died within 48 hours, and the remainder survived until they were killed after 96 to 116 hours. No mortality occurred in 53 young mice or 50 old mice that received 10^4 *E coli*/g, nor were there any deaths among 17 mice after injection with 1 μL/g of PBS.

Effect of bacterial challenge (10^4 CFU/g) on circulating and marrow neutrophils. Four noninfected young mice displayed blood neutrophil concentrations of 1.6 × $10^3/\mu$L (0.7 to 2.1) (mean and range); four old controls exhibited neutrophil concentrations of 2.4 × 10^3 (2.0 to 2.9) ($P = .057$). The peak concentration of neutrophils was observed 2 hours after inoculation for both groups; four young mice had a peak of 3.3 × 10^3 (2.4 to 4.6) neutrophils per μL; four old mice exhibited a peak of 6.2 × 10^3 (3.3 to 11.3)/μL ($P = .057$). After 6 hours, the neutrophil concentrations for both groups had returned to their control ranges.

The mean value for the marrow's NSP (metamyelocytes plus band plus segmented neutrophils) of noninfected old mice was 47% larger than that for young control animals (Fig 1). After bacterial inoculation, the NSP diminished to a similar absolute value for both groups (Fig 1). After 4 hours, however, the NSP of old animals was depleted by a mean of 12.8 × 10^6 cells/two femurs (59% depletion); whereas the young animals experienced a smaller depletion: a mean of 6.4 × 10^6 cells/two femurs (43% depletion). Twenty-four hours after inoculation, the NSP of young animals had begun significant repletion; by 48 hours, the number of cells in the NSP had returned to the range of control values. In contrast, the NSP of old animals remained significantly smaller than that of noninfected controls, and was not repopulated even after 48 hours ($P < .005$).

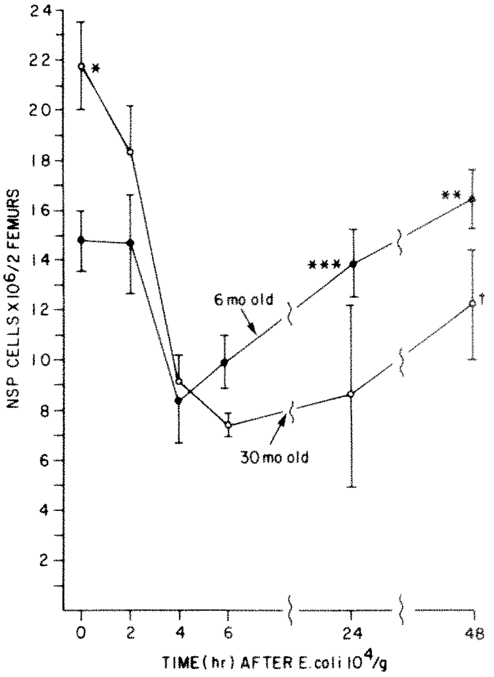

Fig 1. Effect of *E coli* inoculation on the neutrophil storage pool. Means for groups of four to seven 30-month-old mice (○) and for four to eight 6-month-old mice (●). Brackets indicate SE. *$P < .01$ *v* 6-month-old controls; **$P < .005$ *v* 6-month-old mice at 4 hours; ***$P < .03$ *v* 6-month old mice at 4 hours; and †$P < .005$ *v* 30-month-old controls.

Four hours after inoculation, the pool of morphologically recognizable proliferative precursors (blasts, promyelocytes, and myelocytes) was unchanged in young animals, but after 48 hours had increased to a mean of 145% of that for controls ($P < .02$) (Fig 2). In contrast, after 4 hours, the proliferative pool of old animals was reduced to a mean of 56% of the control value ($P < .05$) and remained depressed at 24 hours (Fig 2). Forty-eight hours after inoculation, old animals exhibited repletion of the proliferative pool to control values. As controls, 11 old mice and six young mice received injections of 1 μL PBS/g and did not exhibit neutrophilia or alterations in the marrow cellularity.

Changes in the number and proliferative rate of CFU-GM. In young mice, the femoral pool size of CFU-GM was unchanged at 4 hours but after 48 hours, had increased to a mean of 145% of the control value (Fig 3). In old mice, the mean number of CFU-GM fell to 46% of the control after 4 hours and remained reduced (55% of control) at 48 hours.

The thymidine suicide of CFU-GM for eight young control mice was 27% ± 3%, and for 6 old control mice it was 39% ± 4% ($P = .054$) (Fig 4). Thereafter, bacterial chal-

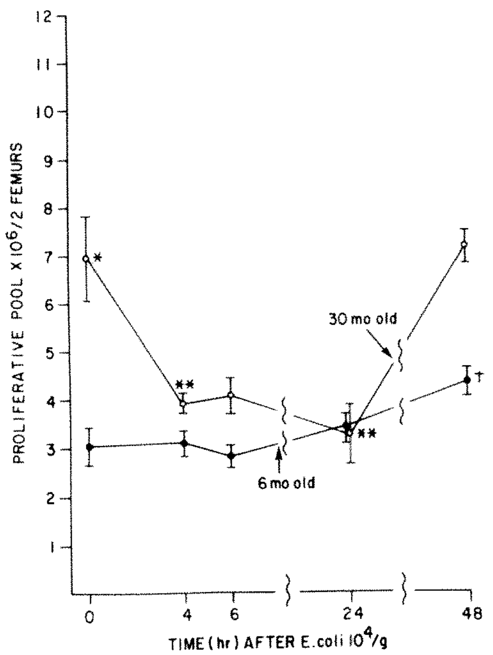

Fig 2. Effect of *E coli* inoculation on the proliferative pool of granulocytes (blasts, promyelocytes, and myelocytes). Means for groups of four to seven 30-month-old animals (O) designate the means for groups of four to eight 6-month-old animals. Brackets indicate SE. $^*P < .003$ *v* 6-month-old controls; $^{**}P < .05$ *v* 30-month-old controls; and $†P < .02$ *v* 6-month old controls.

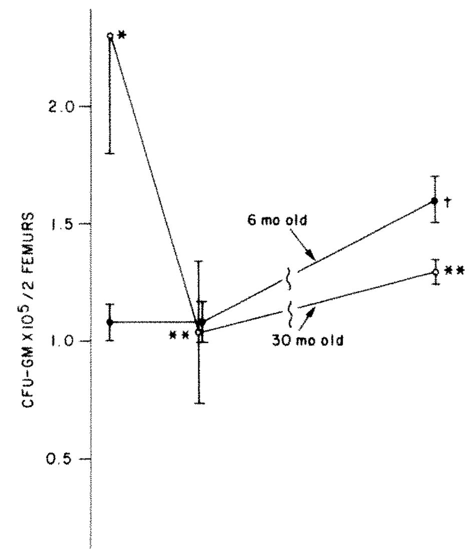

Fig 3. Effect of *E coli* inoculation on the number of CFU-GM. Mean values for groups of four to seven animals, aged 30 months (O); mean values for groups of four to eight 6-month-old mice (●). Brackets indicate SE. $^*P < .04$ *v* 6-month-old controls; $^{**}P < .05$ *v* 30-month-old controls; and $†P < .08$ *v* 6-month-old controls.

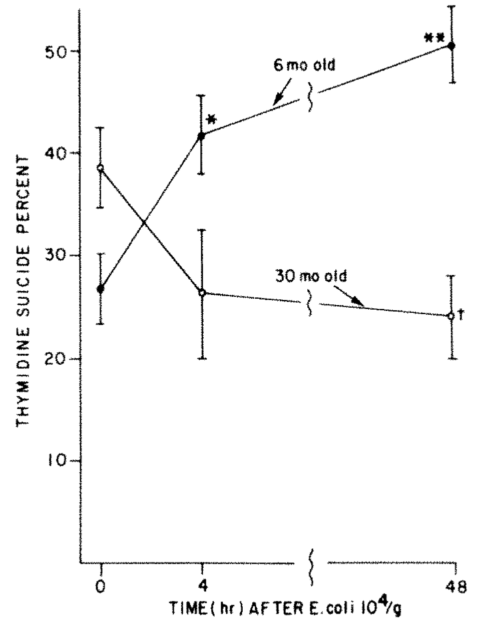

Fig 4. Effect of *E coli* inoculation on thymidine suicide of CFU-GM. Means for groups of four 30-month-old animals (O); mean values for groups of four 6-month-old animals (●). Brackets indicate SE. $^*P < .04$ *v* 6-month-old controls; $^{**}P < .002$ *v* 6-month-old controls; $†P < .04$ *v* 30-month-old controls, $< .002$ *v* 6-month-old at 48 hours.

lenge induced distinctive and different alterations in the thymidine suicide of CFU-GM from the two groups. After 4 hours, the mean thymidine suicide of inoculated young animals had risen ($P < .04$), achieving a peak mean value of 51% ± 4% at 48 hours ($P < .002$ *v* control). In contrast, in old animals, the thymidine suicide of CFU-GM fell within 4 hours and at 48 hours was still depressed as compared with that of 30-month-old controls ($P < .04$) or 6-month-old animals at 48 hours ($P < .002$).

Next, the effect of a larger, ~50% lethal, bacterial inoculum was tested. Twenty-one young mice were given 10^5 *E coli*/g; 10 died within 48 hours. Six old mice received the same weight-standardized dose and three died within 48 hours. Forty-eight hours after inoculation, CFU-GM from four young survivors and three old survivors were evaluated (Table 1). Young mice responded to the larger inoculum with an even greater increase in the number of CFU-GM as well as an increase in the thymidine suicide of CFU-GM. In contrast, old mice displayed a reduced population and thymidine suicide of CFU-GM. In aged mice, the high dose of bacteria induced a significant further decrement in the number of CFU-GM as compared with the smaller nonlethal dose of 10^4/g ($P < .05$) (Table 1).

Morphological studies of the progeny of CFU-GM in vivo. Twenty-eight to 38 colonies were aspirated from 7-day-old cultures and examined morphologically. In this way, the proportion of neutrophils within colonies was determined for groups of three to four old or young animals. The data for control animals and for animals who had received *E. coli* 48 hours previously are shown in Table 2. With bacterial challenge, young animals significantly decreased the propor-

Table 1. Effect of *E coli* Inoculation on Number and Thymidine Suicide Rate of CFU-GM

Mice	CFU-GM × 10^5/2 Femurs	Thymidine Suicide (%)
6 months old		
Control (8)	1.1 (0.7-1.4)	27 (19-42)
10^4/*E coli*/g [7]	1.5 (1.1-1.9)*	51 (31-61)*
10^5 *E coli*/g [4]	1.9 (1.5-2.1)†	49 (43-53)*
30 months old		
Control (6)	2.3 (1.0-3.9)	39 (28-55)
10^4/*E coli*/g [4]	1.3 (1.2-1.5)*	24 (18-36)*
10^5/*E coli*/g [3]	1.0 (0.8-1.1)‡	28 (25-29)*

Means are given for control mice and those injected with *E coli* 48 hours earlier. Ranges are shown in parentheses. Numbers of experiments in each group are shown in brackets.

*$P < .05$ *v* control.

†$P < .05$ *v* value for 10^4/g *E coli.*

‡$P = .028$ *v* 10^4 *E coli*/g 30-month-old mice and 10^5 *E coli*/g 6-month-old mice.

tion of colonies devoid of neutrophils. In contrast, in old animals, the mean number of colonies with no neutrophils nearly doubled (from a control value of 24% to 46%). In addition, the proportion of colonies containing 34% to 100% neutrophils declined from 34% (control) to 11% in old animals inoculated with 10^5 *E coli*/g ($P < .05$), but did not change in the young mice.

DISCUSSION

In noninfected animals and humans, the production and utilization of neutrophils are balanced, so that relatively constant pools of proliferative and stored mature neutrophils are sustained.[7] During infection, the system is perturbed. Cells are acutely released from the NSP into the blood, increasing the blood neutrophil pool and decreasing the size of the NSP.[1,2] In compensation, there is an increased proliferative effort, and the neutrophil supply is restored.[2] These kinetic and proliferative responses depend on an intact system of regulatory stimulators (or inhibitors) and target cells capable of an appropriate response. The present studies were carried out on the premise that during perturbation of granulocyte kinetics by a standardized infectious challenge, the regulation of neutrophil kinetics and proliferation could be evaluated in groups of young and aging animals.

Table 2. Morphology of CFU-GM Colonies in Control Mice and Mice Inoculated With *E Coli* 48 Hours Previously

	6 Months Old			30 Months Old		
Neutrophils in Colonies (%)	Control (36)	*E coli* 10^4/g (33)	*E coli* 10^5/g (33)	Control (38)	*E coli* 10^4/g (32)	*E coli* 10^5/g (28)
0	25%	18%	6%*	24%	31%	46%†
1-33	42%	42%	58%	42%	57%	43%
34-66	19%	25%	21%	18%	9%	4%‡
67-100	14%	15%	15%	16%	3%	7%

Values are for proportion of colonies in each category; numbers of colonies evaluated for the respective categories are shown in parentheses.

*$P < .05$ *v* 6-month-old controls or 30-month-old 10^5/g.

†$P < .05$ *v* 30-month-old controls, $P < .001$ *v* 6-month-old 10^5/g.

‡$P < .03$ *v* 6-month-old 10^5/g.

First, we examined pool sizes and proliferation in the steady state. Femoral marrow was studied quantitatively, and the data were taken to reflect total marrow cellularity, since previous measurements indicate a similar marrow distribution in young and old mice.[3,8,9] The total marrow cellularity of old mice was greater than that of young animals. These data agree with those of Boggs et al.[10] Williams and co-workers also noted an increase in marrow cellularity in old mice, but this difference was not apparent when values were adjusted for the animals' body weight.[9] In our experiments, the old mice also exhibited an increased NSP, an increased proliferative pool, and an increased pool of CFU-GM. None of these increases were due to differences in body mass, because young and old animals did not differ in weight. In addition, old mice exhibited an increased thymidine suicide of CFU-GM. Because the time for DNA synthesis is fixed in mammalian cells,[11] an increase in thymidine suicide is considered a reflection of a shortened cell cycle duration and therefore an increased rate of proliferation. Thus, baseline neutrophil production may be greater in old mice as compared with young adult mice.

After a nonlethal challenge with *E coli* (10^4/g), young mice exhibited a reduction in the NSP and neutrophilia as neutrophils were released into the blood. Then the population of immature granulocytes and of CFU-GM increased, and the thymidine suicide rate of CFU-GM also increased. The increase in proliferative effort is the consequence of stimulus-driven granulocytopoiesis, which facilitates repopulation of the NSP.

Old mice exhibited a greater degree of neutrophilia than did young animals. Esposito and Pennington noted similar degrees of neutrophilia in 24-month-old and 6- to 8-month-old mice challenged with bacteria although the older mice exhibited slightly but consistently lower values.[12] We are hesitant to compare our results with theirs, however, because in the present study older mice and a different organism were used. In our experiments, inoculated old mice lost a greater number of neutrophils from their NSP, and the proportional depletion of the NSP was also greater. These data reflect an intact stimulus and response for neutrophil release, but one resulting in a greater degree of depletion of the NSP in old mice. Even so, neutrophil supply was not sufficiently compromised at the dose of 10^4/g to induce neutropenia. Neutrophil counts were not measured after the high dose of *E coli.* Kinetically, the exaggerated depletion of the NSP is the net consequence of inflow of neutrophils to the NSP from the mitotic compartment and exit of cells into the blood. We did not directly measure neutrophil release, but our studies suggest that diminished proliferation contributes to the exaggerated depletion and to delayed repletion of the NSP. In support of this, the population of immature granulocytes fell acutely in old animals and then returned to control levels rather than remaining stable and then rising as it did in young mice. The size of the pool of immature granulocytes was undoubtedly influenced by movement of immature cells into the NSP. This was accompanied by a reduction in the number of CFU-GM however, suggesting that reduced inflow into the immature cell pool was also a factor. The significance of a reduced pool of CFU-GM appears to be compounded by a reduction in the differentiation of CFU-GM to the neutrophil series in vitro. In contrast, in inoculated young mice, differentiation to neutrophils was increased. An even more pronounced fall in the number of CFU-GM was observed in old mice given the high dose of 10^5 *E coli*/g, whereas the high dose of *E coli* yielded a further increase in CFU-GM in young mice. The reduction in the pool of GFU-GM was not accompanied by an increase in the proliferative rate of CFU-GM, which actually fell after both the high and low doses of *E coli*. Therefore, inoculated old mice did not respond with increased granulocytopoiesis. Although the mechanisms responsible for this are not clear, candidate explanations include insufficient production of a necessary stimulator, production of an inhibitor of proliferation, or hyporesponsiveness of precursors to stimulators.

It has been proposed that during aging, mitotically capable cells lose their ability to proliferate. Hayflick has demonstrated loss of replicative potential in senescent diploid cell lines,[13-15] and Dell'Orco et al correlated the life span of fibroblasts with their number of cell doublings.[14] In addition, Albright and Makinodan showed that hematopoietic stem cells from old animals produced relatively few spleen colonies in irradiated recipients.[17] Lipschitz et al demonstrated reduced numbers of CFU-GM, BFU-E, and CFU-E in elderly anemic subjects,[18] and reduced iron incorporation by aged murine erythroid cells in vitro[19] and in vivo.[20] On the other hand, Boggs et al did not identify a proliferative defect in aging cells used to repopulate W/W^v mice,[21] but reported a sluggish repair of anemia in phlebotomized aged mice.[22] Williams et al reported a reduction in the numbers of BFU-E, CFU-E, and CFU-GM in 42-month-old (but not 6-month-old) mice caged in groups.[9] This abnormality does not appear to be restricted to aging mice, however, since other researchers have reported a reduction in hematopoietic spleen colonies derived from 3- to 5-month-old mice housed in groups.[23] The kinetics of the neutrophil response and its effect on proliferation have not been evaluated in group-housed mice, and the factors responsible for depressing precursors are not at all clear. In the present study, mice were shipped and housed singly. Despite these precautions, we cannot exclude the possibility that factors such as shipping (or other factors unknown to us) may have influenced or primed the hematopoietic system of aging mice, thus contributing to their response to infection.

We applied a standardized stimulus to evaluate neutrophil kinetics in bacterially challenged old mice. The response of old mice differed from that of young animals, and was characterized by (a) an exaggerated depletion of the NSP, (b) a paradoxical diminution of the pool sizes of CFU-GM and their progeny, (c) a paradoxical lack of accelerated proliferation of CFU-GM, and (d) a diminished differentiation of CFU-GM to neutrophils. These changes reflect an absence of the expected stimulus-driven granulocytopoiesis which appropriately increases the neutrophil supply in young mice. As yet, the effect of this abnormality on host resistance is unknown, although in the present short-term experiments, no increase was observed in the mortality of infected aged animals. We propose that the model of bacterially challenged aged mice may be useful in identifying the mechanisms responsible for these abnormalities.

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Homing Receptors on Human and Rodent Lymphocytes—Evidence for a Conserved Carbohydrate-Binding Specificity

By L.M. Stoolman, T.A. Yednock, and S.D. Rosen

Lymphocyte recirculation begins with the attachment of circulating cells to the structurally distinctive postcapillary venules of lymphoid organs termed high-endothelial venules (HEVs). In both rodents and humans, the attachment of lymphocytes to the HEVs of peripheral lymph nodes (PNs) on the one hand and gut-associated lymphoid tissues (GALTs) on the other appears to involve discrete adhesive structures on the surfaces of the interacting cells. In rodents, we previously showed that a carbohydrate-binding receptor at the lymphocyte surface participates in the attachment to the HEV of peripheral nodes. The studies reported herein document the involvement of a similar receptor in the selective attachment of human peripheral blood lymphocytes to the HEVs of PNs. We argue that the close functional relationship between the human and rodent receptors indicates that this component of the adhesive interaction has been conserved through evolution.

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LYMPHOCYTES continuously migrate from the bloodstream into lymphoid organs. This process distributes normal lymphocytes throughout the lymphoid system and may facilitate the dissemination of lymphoid malignancies. In peripheral lymph nodes (PNs) and gut-associated lymphoid-tissues (GALTs) (ie, Peyer's patches or appendix), transvenular migration begins with the attachment of circulating lymphocytes to postcapillary venules with a distinctive cuboidal endothelium (the high endothelial venules or HEVs).

Several lines of evidence indicate that this adhesive interaction is organ selective. In the rat, monospecific antisera have been developed that block the attachment of recirculating lymphocytes to the HEVs of either PNs or GALTs.[1,2] In the mouse[3] and human[4] systems, T cells as a population show preferential binding to the HEVs of PNs whereas B cells bind preferentially to the HEVs of GALTs. In addition, murine lymphomas have been identified that bind to the HEVs of PNs or GALTs with nearly absolute fidelity.[5] These data suggest that the attachment of lymphocytes to the HEVs of PNs on the one hand, and GALTs on the other involve different adhesive structures on the surfaces of the interacting cells.

Candidate adhesion molecules on the lymphocyte surface (referred to as homing receptors) have been isolated from rodents. In mouse, a ubiquinated glycoprotein (gp) termed gp 90mel-14 appears to be the PN-homing receptor.[6-8] In the rat, distinct gps with PN-HEVs and GALT-HEVs binding activities have been detected in thoracic duct lymph. Considerable evidence suggests that these factors are shed lymphocytic homing receptors.[9,10]

We have been interested in the biochemical basis of the adhesive interaction between lymphocytes and the HEVs. Previous work indicates that a carbohydrate-binding receptor on the lymphocyte surface participates in the attachment of rodent lymphoid cells to PN-HEVs in vitro[11,12] and in vivo.[13] In the mouse, this receptor is either identical or closely related to gp 90 Mel-14.[14] In addition, we have shown directly that carbohydrate moieties on the PN-HEVs are required for lymphocyte attachment.[15] These results support the hypothesis that organ-selective migration across the PN-HEV begins with the binding of a lectin-like receptor on lymphocytes to a carbohydrate-containing ligand on the endothelial surface.

In this article, we present evidence that the attachment of human peripheral blood lymphocytes to PN-HEVs also involves a carbohydrate-binding receptor on the lymphocyte surface. The binding specificity of the receptor is similar to its counterparts on rodent lymphocytes. The preservation of carbohydrate binding specificity across species lines suggests that the recognition/binding domains of the adhesive structures on lymphocytes and HEVs have been conserved through evolution.

MATERIALS AND METHODS

Chemicals

Heparin (H3125), chondroitin sulfate (C3254), hyaluronic acid (H7630), all phosphorylated monosaccharides, bovine serum albumin (BSA) (A7906), trypsin type III (T8253), borax (B9876), boric acid (B0252), cyanogen bromide (C6388), cacodylic acid (C0250), disodium EDTA (ED2SS), Histopaque (1077), poly-L-lysine (P1524), clostridial neuraminidase (2133), and paraformaldehyde (P6148) were obtained from Sigma Chemical (St Louis). PPME, the polyphosphomonoester core from *Hansenula hostii* phosphomannan,[16] was the generous gift of Dr M. E. Slodki (U.S. Department of Agriculture, Northern Regional Research Center, Peoria, IL). Lyophilized thrombin was obtained from Parke-Davis (Ann Arbor, MI). Fluorescent microspheres (0.6-μm, FX, 112030-30) were obtained from Duke Scientific, (Palo Alto, CA). Minimal essential medium (MEM) (330-1435) and tricine (845-1398) were purchased from GIBCO Laboratories (Grand Island, NY). Powdered FA buffer was obtained from Difco laboratories (Detroit).

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Mouse anti-human monoclonal antibodies and FITC-labeled goat-anti-mouse IgG were obtained from Coulter Corporation (Hialeah, FL).

Preparation of Cell Suspensions

Rat cells. Lymphocytes were obtained from the cervical and mesenteric nodes of Sprague-Dawley rats (female, 180–200 g) after killing by CO_2 anesthesia and cardiac puncture. Thymocytes were obtained from the thymus after dissection away from parathymic lymph nodes. Cells were gently teased free of connective tissue, collected into MEM buffered with 40 mmol/L tricine (pH 7.4) and supplemented with 1 mg/mL BSA or 5% fetal calf serum (FCS) (assay buffer), washed, and resuspended as described for each procedure. All steps were carried out at 4°C.

Human cells. Peripheral blood mononuclear cells (PBMCs) were fractionated from the peripheral blood of healthy volunteers using a discontinuous gradient of either Ficoll-Hypaque[17] or colloidal silica (Sepracell-MN; Sepratech, Oklahoma City). This procedure removed the bulk of granulocytes, RBCs, and platelets.

Because excessive contamination by platelets resulted in cell clumping during the in vitro binding assay, the cell suspensions were purified further using one of two methods: (a) sequential 200 g, 10 minutes, 4°C centrifugations in calcium, magnesium-free PBS (CMF-PBS), or (b) brief treatment with thrombin as follows. The mononuclear suspension prepared from 60 mL blood was diluted 1:1 with Hank's balanced salt solution (HBS) and centrifuged (~400 g, 15 minutes). The pellet was resuspended in 2 mL of HBS; 10 μL of thrombin solution [1:20 to 1:100 dilution of frozen stock (100 U/mL) in HBS] were then added to 1-mL aliquots of the cell suspension at room temperature. The suspensions were gently agitated (~30 seconds) and centrifuged briefly at 13,000 g to pellet the platelet–fibrin clumps. The mononuclear cells remaining in suspension were pelleted (400 g, 2 minutes, 4°C), the supernatant was discarded, and the cells were resuspended in buffer containing 5% heat-inactivated FCS. Indirect immunofluorescence with T11 (pan-T cell), B1 (pan-B cell), and M01 (monocyte and granulocyte specific) mouse–anti-human monoclonals indicated that PBMC suspensions prepared with either method contained >90% lymphocytes, predominantly mature T cells (data not shown).

T cell lines, isolated after allogeneic stimulation of PBMCs, were provided by Dr Bruce Richardson (Department of Internal Medicine, University of Michigan). Lines were maintained in RPMI 1640 supplemented with 10% calf serum and interleukin 2 (IL 2), pH 7.4, with restimulation by irradiated allogenic PBMCs at 2-week intervals. Cells were harvested during log-phase growth, washed, and resuspended in assay buffer.

HEV binding assay. The binding of lymphocytes to the HEVs of rat lymph nodes was determined essentially as described previously.[12] In brief, suspensions of rat or human lymphocytes were preincubated (15 minutes, 4°C) in the presence of test substances or buffer alone. The suspensions (10×10^6 cells/mL unless otherwise specified) were then applied to paraformaldehyde-fixed, frozen sections of rat cervical lymph nodes and gyrated (60 rpm) at 7 to 10°C for 30 minutes. The cell suspensions were then gently decanted, and the sections were fixed in glutaraldehyde (3% in PBS, 20 minutes, 4°C) and stained with toluidine blue. In some experiments, sections were dipped in PBS several times prior to staining to remove unattached cells. The affinity of lymphocytes for HEVs was determined by measuring the average number of lymphocytes bound to HEVs per 200× microscopic field. The binding after experimental treatment is expressed as the percentage of binding relative to that in the control (cells exposed to buffer alone). This method of analysis facilitated the pooling of data from independent experiments conducted under the same conditions. SEM for this procedure varied from 10% to 30%.

The binding of human lymphocytes to the HEV of human lymph nodes was determined as above except that PBMCs were routinely preincubated with neuraminidase (0.005 U, 30 minutes, 37°C) before binding assays were conducted. This treatment enhanced the adhesive interaction between human lymphocytes and human HEVs without altering specificity (described in the Results section). Human peripheral lymph nodes were snap-frozen in OCT and stored at −70°C until use (2 weeks to 2 months). Only surgical biopsy specimens showing normal or hyperplastic histology were used in the binding assay.

PPME-bead assay. The conjugation of PPME, the polyphosphomonoester core polysaccharide derived from the yeast *Hansenula holstii,* to fluorescent microspheres and the procedure for measuring the attachment of conjugated beads to lymphocytes has been presented in detail elsewhere.[18] In brief, 3 to 7 mg PPME was reacted with cyanogen bromide (100 mg/mL) in distilled water. The pH of the reaction was monitored and maintained at ~11 by the dropwise addition of NaOH (0.5 mol/L). The cyanogen-bromide–activated PPME was separated from free cyanogen bromide over a G50 Sephadex column in borate buffer (0.2 mol/L, pH 8.0, room temperature). The 2 to 3 1-mL fractions with the highest concentration of carbohydrate (determined by the phenol-sulfuric assay) were pooled, diluted 1:10 with borate buffer, and added to 0.6 μm microspheres (100 μL/1 mL PPME solution). After two-24 hour incubations at room temperature, the conjugated beads were washed in distilled water, aliquoted, snap-frozen in liquid nitrogen, and stored at −70°C until use. Conjugated beads prepared in this manner may not withstand long-term storage. Some preparations lost cell binding activity completely within 2 weeks. Therefore, experiments were conducted with freshly thawed aliquots within 7 to 10 days of preparation.

Prior to use, the beads were sonicated to disrupt clumps, washed, and resuspended in assay buffer. The binding assay was initiated by adding 50 μL PPME-beads to 5×10^5 cells in flat-bottomed, 96-well microtiter plates (200 μL final volume; beads diluted 1:32 relative to stock solution). The plates were centrifuged and then allowed to incubate undisturbed for 60 minutes on ice. Cells with attached beads were resuspended, separated from free beads by centrifugation through a 6% BSA solution, and fixed in 1% paraformaldehyde.

Cell-associated fluorescence was quantified on a Coulter Epics V flow cytometer. A narrow low-angle light-scatter gate, set on a control population of cells without attached beads, excluded both cell clumps and free beads from the analysis. A wide 90° light-scatter gate was set to assure that cells with large numbers of attached beads would not be excluded from the analysis. Fluorescence histograms of 5 to 10,000 cells, exposed to either PPME-conjugated or nonconjugated beads, were collected. The fluorescent signal in the latter population was taken as a measure of nonspecific bead attachment and subtracted from the signal measured with PPME-beads (EASY analytic software, Coulter Corp, Hialeah, FL).

The binding of beads to lymphocytes was characterized by measuring both the percentage of fluorescing cells (those with one or more beads attached) and the amount of fluorescence per cell (a measure of the number of beads per cell). Perturbation of the adhesive interaction altered one or both of these parameters. Therefore, a unitless measure of specific bead binding—the product of the percentage of fluorescing cells and the mean fluorescence channel number for the entire population (SBI)—was calculated for each experimental condition. The degree of bead binding in experimental samples was expressed relative to that in control samples (cells exposed to conjugated beads in the absence of additives).

Cell Treatments

Trypsin. Cells were harvested, washed, and resuspended in Dulbecco's PBS. The trypsin digestion was conducted in PBS (10 minutes at 37°C). The reaction was terminated by the addition of excess cold assay buffer. The cells were washed and resuspended in assay buffer and used immediately in binding assays.

EDTA. Prior to the HEV and bead binding assays, cells and beads were washed and resuspended in MEM containing 2 mmol/L disodium EDTA with or without an additional 3 mmol/L calcium or magnesium chloride. The HEV binding assays were initiated by adding cell suspensions directly to tissue sections. The bead-binding assay was initiated by combining cells and beads as described above.

Carbohydrates. Cell suspensions were incubated (15 minutes, 4°C) with carbohydrates prior to the start of the HEV or bead-binding assays. The HEV assays were initiated as described above. Carbohydrates were present throughout the incubation period at the concentrations specified for each experiment.

Neuraminidase. Gradient-purified human PBMCs were incubated (1×10^7 cells/mL, 30 minutes, 37°C) with neuraminidase (0.005 U final concentration in CMF-PBS). The suspensions were subsequently washed in cold assay buffer, resuspended at 5 to 10×10^6 cells/mL, incubated 15 minutes on ice, and then used in lymphocyte-HEV binding experiments. Enzyme treatments were conducted immediately after gradient separation and before exposure to potential inhibitors of the lymphocyte binding interactions (eg, carbohydrates or EDTA).

RESULTS

The adhesive interaction between human lymphocytes and the HEVs of lymph nodes from humans and rats were examined using an in vitro assay adapted from the work of Stamper and Woodruff.[19,20] We used rat PNs as the routine source of HEVs since previous reports indicated that rodent tissues can substitute for analogous human tissues in the in

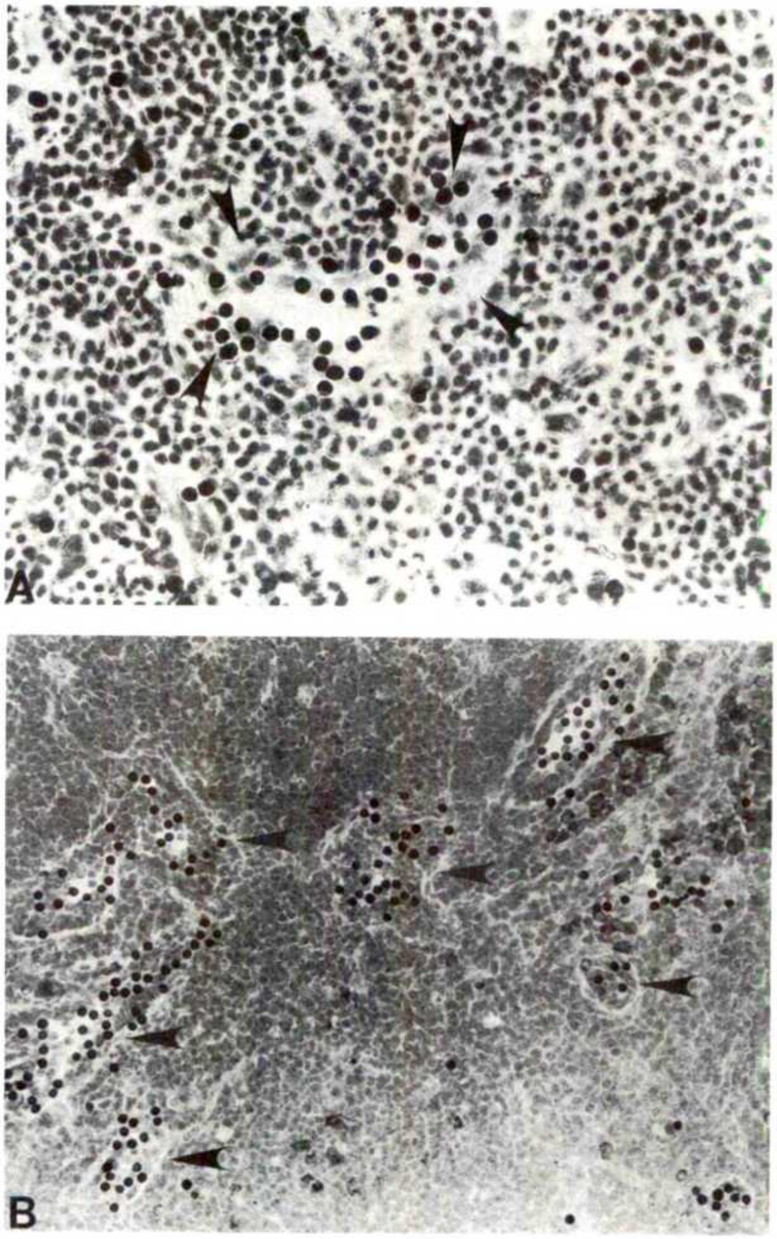

Fig 1. Human PBMCs adhering to the HEVs of human (A) and rat (B) peripheral lymph nodes. Exogenous PBMCs (dark cells 7 to 10 μm in diameter) bind to HEVs (vascular structures with defined lumena and prominent lining cells). In contrast to the HEVs, rat sinusoids (not shown) bind significant numbers of both erythrocytes and lymphocytes, indicating that adhesion to this structure is less specific.

vitro adhesion assay.[21,22] Nonetheless, critical experiments were performed with both human and rat lymph nodes as targets.

Human lymphocytes bound avidly and specifically to the HEVs of paraformaldehyde-fixed, frozen sections of both human and rat lymph nodes (Fig 1A and B). The density of binding over the HEVs was at least 50-fold greater than that to most other regions. This binding interaction could be enhanced by pretreating PBMCs with neuraminidase (Table 1). Such pretreatment did not alter the specificity of inhibitors such as mannose-6-phosphate (M6P) or EDTA (data not shown); it simply increased the baseline density of lymphocyte binding to the HEV.

When tested in parallel at the same cell concentrations, human PBMCs bound 50% to 70% as well as rat nodal lymphocytes to rat HEVs. The magnitude and reproducibility of the binding to rat HEVs far exceeded that to human material, however, and may reflect the lability of the binding sites on HEVs. Rat lymph nodes were snap frozen immediately on removal from the animal and used within hours. Human material could not be frozen as expeditiously, and several weeks generally elapsed before tissues were available for experimental use.

As previously described for rodent lymphocytes, the binding of human lymphocytes to rat HEVs was calcium dependent and trypsin sensitive (Fig 2). Chelation of divalent cations with 2 mmol/L EDTA reduced binding by 70%, the addition of 3 mmol/L Mg^{2+} increased attachment to a slight degree whereas the same concentration of Ca^{2+} returned binding to control levels. A 10-minute preincubation of lymphoid cells with 20 μg/mL trypsin reduced binding by 70% whereas 40 μg/mL eliminated binding completely.

The capacity to attach to rat HEVs was not a characteristic of all human lymphocytes examined. Two IL 2-dependent, T4-positive lymphoid lines failed to bind (Fig 2). In contrast, Navarro et al reported that IL 2-dependent T cell lines bound to human PN-HEVs.[23] This discrepancy may reflect the fact that we examined interspecies rather than intraspecies adhesion. Alternatively, the lines we used may be at a stage of differentiation or proliferation at which the homing receptors were not expressed. Such a relationship has been demonstrated in the murine system. Specifically, immature lymphocytes isolated from the thymus,[24] B lymphoblastoid cells isolated from germinal centers of lymph nodes,[25] and a variety of IL 2-dependent lymphoblastoid cell lines[26] failed to express the Mel 14 antigen, bind to HEVs in vitro, or recirculate in vivo. Therefore, the expression of murine homing receptors appears to be regulated. The nature and extent of such regulation in the human requires further study. Nonetheless, the two human lines were useful negative controls in subsequent experiments.

Table 1. Binding of Human Lymphocytes to Human HEVs

Additive	Neuraminidase Treatment	
	(−)	(+)
None	0.7	2.9
Mannose 1 P	0.3	3.5
Mannose 6 P	0.035	0.8

Human PBMC were isolated from peripheral blood as described in the Materials and Methods section. After preincubation (with or without 0.005 U *Clostridium perfringens* neuraminidase as described in the text), 5 × 10^6 cells/mL were layered on fixed, frozen sections of human peripheral lymph nodes. The mannose-phosphates were present at a concentration of 5 mmol/L. Cell binding to HEVs is reported as the number of cells attached to HEVs per 200× microscopic field. Neuraminidase treatment also enhances the binding of human PBMCs to HEVs of rat lymph nodes (data not shown).

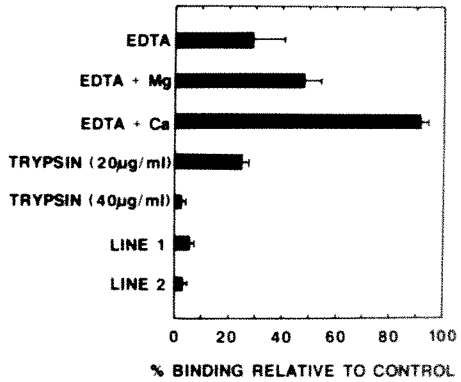

Fig 2. Binding of human PBMCs to rat HEV–divalent cation requirements, trypsin sensitivity, and the interaction with two T cell lines. Three independent experiments are shown. The first three bars show the effects of EDTA (2 mmol/L in assay buffer) with or without additional magnesium or calcium chloride. The next two bars demonstrate the effect that brief trypsinization of the lymphocyte has on their binding activity. The last two bars show the binding activity of two T4-positive T cell lines collected during log phase growth. Data in each of these experiments are expressed as binding relative to untreated PBMC in assay buffer alone. Mean and SEM for each data point were based on four replicate samples.

We next examined the effects of various monosaccharides and polysaccharides on the human PBMC-HEV binding

Table 2. Binding of Human Lymphocytes to HEVs: Inhibition by Carbohydrates

Carbohydrate	Binding Relative to Control	
	Human	Rat
Monosaccharides		
Mannose 6 P	36 (9.5)	14 (5)
Mannose 1 P	84 (8.5)	40 (10)
Fructose 6 P	—	101 (22)
Fructose 1 P	—	15 (5)
Glucose 6 P	—	64 (7)
Glucose 1 P	—	51 (24)
Polysaccharides		
PPME	11 (3.4)	0.5 (0.25)
Fucoidin	15 (4)	2 (1)
Heparin	89 (20)	85 (22)
Chondroitin sulfate	91 (18)	94 (9)
Hyaluronic acid	—	72 (15)

Human PBMCs were prepared as described in the Materials and Methods section; 1 × 10^7 cells/mL were incubated over sections of either human or rat peripheral lymph nodes, with or without added carbohydrates. All monosaccharides were present at 5 mmol/L concentration. Polysaccharides were at 0.1 μg/mL in experiments with human tissue sections and 1.0 μg/mL in experiments with rat tissue sections. The means and SEM (in parentheses) for binding relative to the buffer control were based on a minimum of four replicates.

interaction using both rat and human HEV as targets. In keeping with previous observations in both the rat and mouse systems,[12,18] the attachment of human lymphocytes was inhibited by M6P, the M6P-rich mannan PPME and the sulfated, fucose-rich polysaccharide fucoidin (Table 2). Mannose-1-phosphate (M1P) and a variety of anionic polysaccharides that contain neither fucose nor mannose residues showed significantly less activity. In addition, fructose-1-phosphate, a structural homologue of M6P, inhibited attachment to rat HEVs whereas fructose-6-phosphate and the glucose-phosphates had minimal activity. Thus, human and rodent lymphocytes appeared to use similar carbohydrate binding receptor(s) to attach to HEVs.

The presence of this receptor was confirmed with fluorescent microspheres covalently linked to PPME (PPME-beads). As shown in Fig 3, PPME-beads bound avidly and specifically to human PBMCs. In general, 45% to 75% of the PBMCs bound to PPME-conjugated beads whereas <5% bound to the nonconjugated beads. The percentages of cells that bound PPME-beads (% POS) and the average number of PPME-beads/cell (MCN) varied considerably from one experiment to the next. This variability may reflect batch-to-batch inconsistencies in the conjugation process, leaching of PPME from the bead surface during storage, or modulation of the receptor on the PBMC surface. Physiologic parameters that may alter the expression of the carbohydrate binding receptor(s) are currently under investigation.

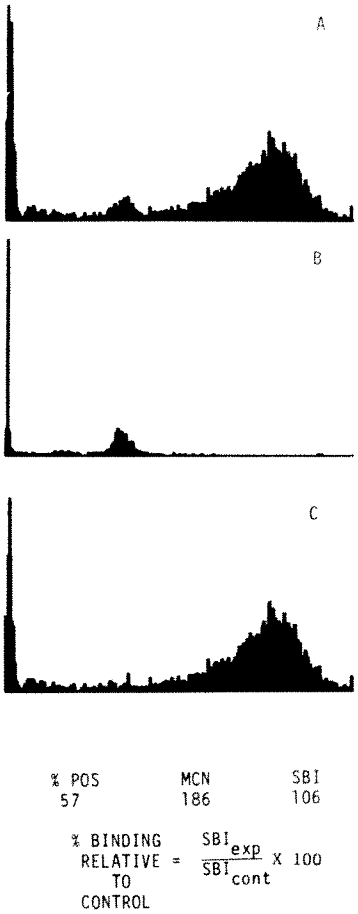

Fig 3. Binding of PPME-conjugated and nonconjugated fluorescent beads to PBMC. (A through C) Fluorescence intensity (256 channels; three-decade log scale) is plotted on the *x*-axis, and the number of cells at each intensity level is registered on the *y*-axis. (A) Distribution of cell-associated fluorescence with PPME-conjugated beads. (B) Distribution of fluorescence with nonconjugated beads. (C) Subtraction histogram (A and B) generated by the IMMUNO analytic software (described in Materials and Methods section). The percentage of the cell population with attached beads (% POS), the mean channel number of the fluorescence distribution (MCN) and the specific binding index (SBI) are calculated from the subtraction histogram; % binding relative to control is the measure used to compare the effects of the various experimental conditions.

The binding of human PBMCs to PPME-beads paralleled the PBMC-HEV adhesive interaction in several respects. First, PPME-bead attachment was calcium dependent and trypsin sensitive (Fig 4). Second, the two T cell lines that failed to attach to rat HEV also failed to express receptors for PPME-beads (Fig 4). Third, the monosaccharide and polysaccharides that inhibited binding to HEV were also effective against PPME-beads (Table 3). Finally, the dose–response curves for the mannose-phosphates, PPME, and fucoidin revealed identical relative potencies in the two adhesion assays. Specifically, M6P was four to fivefold more potent than M1P as an inhibitor of attachment to both HEV and PPME-beads (Fig 5), whereas fucoidin and PPME were equally potent in both assays (Fig 6).

These results parallel those reported by us in mouse[18] and, in Table 4, we report similar findings in the rat. Specifically, the attachment of PPME-beads to rat cervical lymph node lymphocytes was calcium dependent, trypsin sensitive, and inhibited to a greater extent by M6P than M1P. Thymocytes, a nonrecirculating population of cells that do not bind to the HEVs,[26] showed minimal binding to the PPME-beads.

DISCUSSION

In summary, the attachment of normal human PBMCs to PN-HEVs involves a carbohydrate-binding receptor with affinity for M6P and related compounds. Direct analysis, using fluorescent microspheres derivatized with a high-

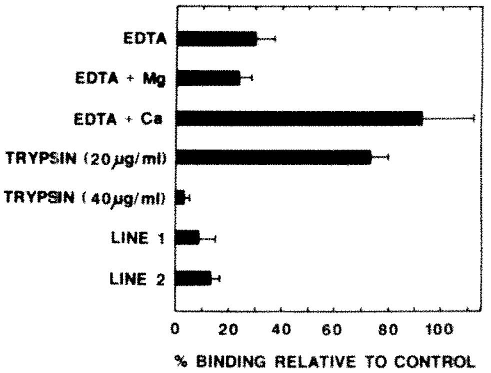

Fig 4. Binding of PBMCs to PPME-conjugated beads–divalent cation requirements, trypsin sensitivity, and the interaction with two T cell lines. The assay was conducted as described in the Materials and Methods section and the legend to Fig 2. Means and SEM were based on a minimum of four replicate samples.

Table 3. Binding of Human Lymphocytes to PPME-Beads: Inhibition by Carbohydrates

Carbohydrate	Binding Relative to Control
Monosaccharides	
Mannose 6 P	19 (5)
Mannose 1 P	97 (22)
Fructose 6 P	96 (3)
Fructose 1 P	35 (7)
Glucose 6 P	91 (5)
Glucose 1 P	86 (6)
Polysaccharides	
PPME	15 (8)
Fucoidin	2 (1)
Heparin	74 (10)
Hyaluronic acid	81 (7)
Chondroitin sulfate	92 (7)

Effects of carbohydrates on the binding of human PBMCs to PPME-conjugated beads. All monosaccharides were present at 5 mmol/L; PPME and fucoidin at 1 μg/mL; heparin at 10 μg/mL; chondroitin sulfate and hyaluronic acid at 100 μg/mL. Means and SEM (in parentheses) were based on a minimum of four replicate samples.

affinity polysaccharide ligand (PPME), reveals that the receptor is functionally similar to those on rat and mouse lymphocytes. Therefore, the attachment of human, rat, and mouse lymphocytes to lymph node HEVs involves a lectinlike receptor on the lymphocyte surface in each species.

Jalkanen et al recently reported antigenic crossreactivity between murine and human PN-homing receptors.[27] In addition, Mel 14 (a monoclonal antibody specific for the PN-homing receptor of murine lymphocytes)[6] blocked the attachment of human PBMCs to the HEVs of both human and murine lymph nodes but not to the HEVs of GALTs. In addition, we recently showed that the MEL 14 antigen is closely linked or identical to the carbohydrate-binding receptor on murine lymphocytes and lymphomas.[14] Therefore, the activity of MEL-14 in the human system further supports the proposal that PN-homing receptors are highly conserved cell-surface lectins.

The fact that the same spectrum of carbohydrates inhibits the attachment of human PBMCs to HEVs from either human or rat peripheral lymph nodes suggests that the ligand for the PN-homing receptor on the HEVs is similarly conserved during evolution. Previous studies have reached similar conclusions. Jalkanen et al isolated EBV-transformed human lymphoblastoid lines that show identical tissue binding specificities toward human and mouse HEVs.[21] Woodruff et al reported a polyclonal rabbit anti-human lymphocyte antibody that blocks the attachment of human PBMCs to the HEVs of human tonsil and rat cervical lymph node.[22] This antibody failed to inhibit the attachment of human PBMCs to rodent mesenteric lymph nodes. Thus, several independent investigations indicate that the organ selective determinants on HEVs are preserved across species lines.

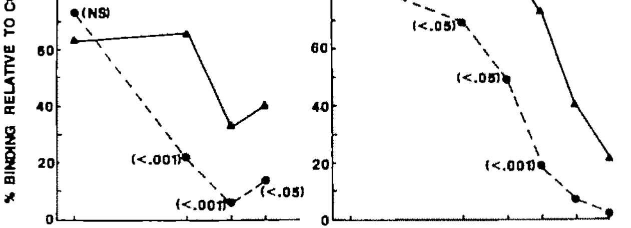

Fig 5. Dose–response curves for mannose phosphates effects on human PBMCs attachment to HEVs (A) and to PPME-conjugated beads (B). Each data point is pooled from 8 to 14 replicates collected from two to four independent experiments. The level of significance (based on Student's distribution) for the differences between M1P (▲) and M6P (●) is recorded in parentheses adjacent to the data points for the latter.

Neuraminidase-treated PBMCs were used to enhance the sensitivity and reproducibility of the human lymphocyte-human HEV binding assay. Although not used extensively in the current study, this treatment similarly accentuated specific binding to both rat HEVs and PPME-beads (data not shown). Therefore, it has become our standard method for conducting all binding assays with human PBMCs.

Mild neuraminidase treatment appeared to enhance adhesion without altering the interaction of the PN-homing receptor with carbohydrate ligands. This is in keeping with the work of Samlowski et al showing that the murine PN-homing receptor functions normally in vivo after neuraminidase treatment ex vivo if one inhibits the sequestration of infused lymphocytes in the liver.[28] Desialation of cell-surface structures may enhance their interactions with specific ligands,[29] as documented in the homotypic interaction of purified neural cell adhesion molecules (N-CAMs) with endogenous N-CAMs on intact cells.[30] In addition, generalized removal of sialic acid residues from the cell surface reduces the net negative charge, resulting in nonspecific accentuation of adhesive phenomena. Therefore, neuraminidase treatment in vitro may directly alter the activity of the lymphocyte homing receptor or indirectly enhance adhesion by reducing surface charge.

The accentuation of lymphocyte binding to HEVs after treatment of the lymphocytes contrasts sharply with the complete inhibition of the binding interaction after neuraminidase treatment of the HEVs.[15] The latter result indicates that sialic acid may be part of the lymphocyte-binding site on the HEVs. Thus, sialic acid moeities on both the lymphocyte and the HEVs can influence their adhesive interaction in vitro. Control of intercellular adhesion through modulation of receptor-associated sialic acid has been proposed for N-CAM[31] based on the correlation between neural cell adhesion and polysialation of N-CAM in brain tissues.[32,33] Whether a similar mechanism regulates the lymphocyte–HEVs interaction in vivo remains to be determined.

The precise relationship of the lymphocyte lectin to adhesive structures such as the Hermes 1 antigen[21] and LFA-1[34] remains to be determined. The Hermes 1 monoclonal appears to recognize an epitope common to the binding sites on lymphocytes for the HEVs of both peripheral lymph nodes and GALTs. The PN-homing receptor precipitated by

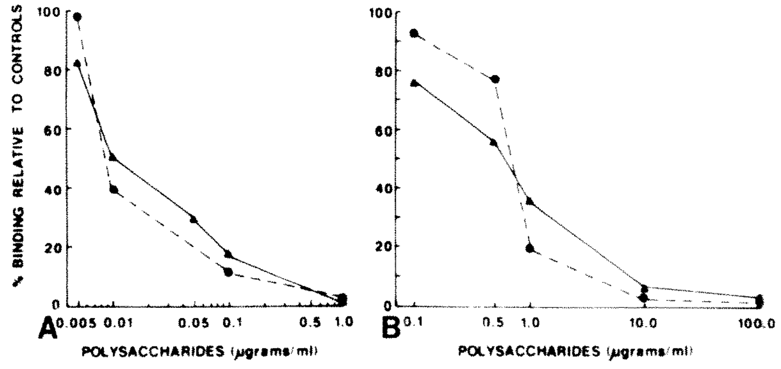

Fig 6. Dose-response curves for PPME and fucoidin effects on human PBMC attachment to HEVs (A) and to PPME-conjugated beads (B). The means of three to six replicate samples are plotted. There was no significant difference between PPME (▲) and fucoidin (●).

Hermes 1 crossreacts with MEL-14 and consists of a single, 90-kd polypeptide, suggesting structural homology between the human and murine structures. In light of the relationship between gp 90 Mel-14 and the murine lectin, we are currently examining the possibility that the Hermes 1 antigen is similarly linked to the human carbohydrate-binding receptor.

LFA-1 participates in a variety of adhesive interactions involving lymphocytes including T cell-mediated cytotoxicity,[35] NK-mediated cytotoxicity,[36] and binding to human umbilical vein endothelium.[37,38] This latter observation has led to speculation that LFA-1 contributes to lymphocyte recirculation and/or migration into chronic inflammatory lesions. Several lines of evidence indicate that LFA-1 is distinct from the cell-surface lectin involved in binding to PN-HEVs. First, binding of lymphocytes to HEVs in vitro is most efficient at 8°C; LFA-1-mediated adhesion is markedly reduced at low temperature.[39] Second, the binding of lymphocytes to PN-HEVs requires calcium, whereas LFA-1-mediated processes are magnesium dependent.[30,39] Third, most murine thymocytes express LFA-1 but neither express the cell-surface lectin nor bind to HEVs. Finally, in S49 murine lymphoma lines, LFA-1 is expressed equally on cell lines with high and low HEV-binding activities.[14] Only the expression of PPME-bead binding sites and Mel-14 antigen correlate with affinity for PN-HEVs. Thus, LFA-1 appears to be distinct from the family of carbohydrate-binding molecules we have described in rodents and humans. Concerted action of the PN-homing receptor and LFA-1 in situ, however, remains an intriguing possibility.

Table 4. Binding of Rat Lymphocytes to PPME-Beads

Cells	Treatment	PPME-Bead Binding
Lymphocytes	Control	100 (28)
	EDTA	18 (1)
	EDTA + Ca^{2+}	70 (12)
	EDTA + Mg^{2+}	24 (5)
Lymphocytes	Control	100 (15)
	M1P	73 (2)
	M6P	17 (6)
Lymphocytes	—	100 (10)
Thymocytes	—	5 (3)
Lymphocytes	Control	100 (5)
	Trypsin	4 (2)
	Trypsin + trypsin inhibitor	94 (14)

Binding of rat lymphocytes to PPME-conjugated beads. The assays were conducted as described in the Materials and Methods section. The analytic procedure for measuring bead attachment differed slightly from the technique used in the above experiments and is described in detail elsewhere.[18] Means and SEM (in parentheses) are based on a minimum of four replicates.

The interactions of PBMCs with PPME-beads and HEVs require Ca^{2+} and show a relatively modest difference in the inhibitory potencies of M6P *v* M1P. These observations contrast with the characteristics of both the high and low molecular weight phosphomannosyl receptors (PMRs) that have been implicated in the intracellular transport of acid hydrolases to the lysosome.[40-44] Recently, both PMRs have been detected in human lymphoid cells and may be expressed at the cell surface.[45] The relationship between these receptors and the PN-homing receptor is currently under study.

The nonspecific inhibition by the glucose phosphates in the HEV-binding assay has been observed with rodent cells[18] and reflects the role of ionic forces in the adhesive interaction. In rodents, increasing the ionic strength above physiologic levels resulted in progressive inhibition of lymphocyte attachment whether the ionic species was sodium chloride or anionic carbohydrates.[11] Furthermore, desulfation of fucoidin and dephosphorylation of PPME eliminated inhibitory activity (L.M. Stoolman and S.D. Rosen, unpublished observations).[12] Inhibition by PPME, fucoidin, M6P, and F1P consistently exceeded that expected on a charge basis alone, however.[12] Thus, biologic activity requires more than the mere presence of negatively charged groups. Perhaps, as has been suggested for the cell-binding domain of fibronectin,[46] the spatial orientation of such groups will prove to be a key factor.

The relationship between the exogenous carbohydrate inhibitors and the endogenous HEV attachment site remains to be defined. Investigation of the murine HEVs indicates that terminal sialic acid residues are critical to the activity of the PN-HEVs attachment site.[15] A more detailed analysis of the PN-HEVs attachment site will be required to determine whether it shares key structural features with either PPME or fucoidin. Such an analysis will also help determine whether the carbohydrate-binding receptor on the lymphocyte attaches directly to the HEVs or, as suggested for ligatin,[47] acts as a membrane attachment site for a ligand that forms an intercellular bridge.

The present study amplifies the work of Butcher et al,[48] who proposed that evolutionary mutations in the interacting adhesive molecules on lymphocytes and HEVs resulted in progressive interspecies incompatibility. They also observed a baseline level of compatibility between widely separated species, however, thus suggesting the persistence of certain essential structural features in the adhesive interaction. The current study indicates that a carbohydrate-binding receptor at the lymphocyte surface is a conserved essential determinant for recognition of PN-HEVs.

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Spontaneous Production of Interleukin 1 Activity by Chronic Lymphocytic Leukemic Cells

By Charlotte Uggla, Miguel Aguilar-Santelises, Anders Rosén, Håkan Mellstedt, and Mikael Jondal

Chronic lymphocytic leukemic B cells (B-CLL) were found to produce an IL 1-like growth factor spontaneously in vitro for mouse thymocytes. This factor was comitogenic with concanavalin A (Con A) and nonmitogenic combinations of phorbol ester and calcium ionophore but not with phytohemagglutinin (PHA). Growth factor production was dose-related to the number of in vitro cultured cells and detectable at 6 hours using high cell concentrations. A small number of admixed normal T cells was not important for factor production. No growth of autologous B-CLL or allogeneic thymocytes was induced by the factor. A chromatographic high-performance liquid chromatography (HPLC) analysis and inhibition experiments with a polyclonal rabbit anti interleukin 1 (IL 1) antiserum indicated that the B-CLL–derived growth factor belonged to the IL 1 family. This was supported by the direct demonstration of IL 1 β in supernatants from B-CLL by radioimmunoassay. Possible biologic implications for B-CLL–derived IL 1 are discussed in relation to tumor cell growth in different clinical stages.

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CHRONIC lymphocytic leukemia of B cell type (B-CLL) is characterized by a clonal proliferation of cells that can differ substantially with regard to malignant behavior.[1-3] Rai et al proposed a clinical staging system (0 through IV) in which stage 0 is characterized by a blood lymphocytosis but no other disease-related findings. In stage IV, the disease is progressive with thrombocytopenia and anemia. The median survival for stage 0 is >8 years whereas stage IV patients survive for 9 months only.[4] The reasons for this wide variability in malignant behavior are not well understood but could be related to both an aberrant T cell control[5,6] as well as to inherent growth characteristics of individual cell populations.[7,8]

Regulation of B cell growth is highly complex and dependent on the combined effect of different factors for initiation and continued maintenance of cell cycling.[9,10] Melchers et al defined three different restriction points in the B cell cycle.[11] The first of these is triggered by antigen through interaction with specific Ig receptors and enters the cell into the G1 phase. The second is located in late G1 and is dependent on monokine factors such as IL 1 (α factors). The third one is positioned in G2 and is triggered by T cell products such as BCGF (β factors). Differences in growth potential between different B-CLL clones could thus be related to both an aberrant growth factor production and an increased tendency of the tumor cells to pass certain restriction points.

Interleukin 1 (IL 1) was originally described as a monokine but has recently been shown to be produced by a number of different cell types.[12] Two different monocyte-derived IL 1 genes have been cloned which code for IL 1α (pI 5.0 and 17 kd) and IL 1β (pI 7.0 and 17 kd) with distinct molecular properties.[13,14] Biologic IL 1 activity has also been found in polypeptides ranging from 2 to 75 kd,[15] the larger being an aggregate form and the smaller cleavage product from a 31-kd precursor molecule. IL 1 induces a multitude of biologic activities such as fever, production of acute phase reactants, coactivation of lymphocytes, chemotaxis of polymorphonuclear (PMN) leukocytes, proliferation of fibroblasts and endothelial cells, and different forms of antitumor cell defense mechanisms.[16]

In the immune response, macrophage-derived IL 1 induces the upregulation of IL 2 receptors on T cells and also acts as a growth factor for B cells. In addition, clonal B cells produce IL 1 in line with their macrophagelike capacity to process and present antigen.[12] Recently, Pistoia et al demonstrated that both normal B cells and some B chronic leukemic cells produce IL 1 in the absence of any apparent stimulus.[17] Some Epstein-Barr virus (EBV) transformed B cell lines produce both IL 1 and "contra IL 1."[18,19] This IL 1 activity may be mediated by factors that differ from monocyte-derived IL 1, as shown by protein sequencing and neutralization studies.[20]

When analyzing growth factors secreted by B CLL cells in vitro, we found no spontaneous production of B cell growth factor (BCGF); BCDF or IL 2 but evidence for IL 1-like factor activity (B-CLL–derived IL 1) in the standard mouse thymocyte assay. The present work further defines IL 1-like activity from a progressive stage IV patient with regard to comitogenicity with different inducers of proliferation, direct growth-inducing properties, and susceptibility to inhibition by polyclonal antibodies. In addition, the presence of IL 1β has been analyzed in supernatants from B-CLL cultures representing different clincial stages.

MATERIALS AND METHODS

Isolation and fractionation of leukemic cells. AD B-CLL were obtained by leukapheresis. Cells were then isolated using the Ficoll-Hypaque one-step density-gradient technique and frozen in 10% dimethylsulfoxide (DMSO) with 20% fetal calf serum (FCS) for later use. Normal lymphocytes present in B-CLL preparations were analyzed using monoclonal antibodies against CD3, CD16, and CD13 and by rosette formation for detection of CD2 receptors. Normal T cells were removed using sheep RBC (SRBC) sedimentation with AET-treated SRBC as described.[21] Additional leukemic

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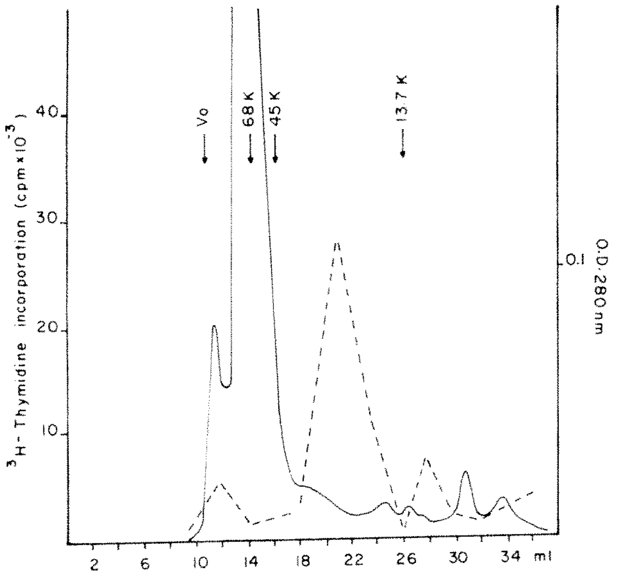

Fig 1. Supernatant material from AD B-CLL culture was concentrated and purified in a two-step HPLC procedure using a hydroxylapatite Biogel HPHT column and an Ultropac TSK-G2000 SW column as described in the Materials and Methods section. Fractions were tested for protein content (——) and induction of direct mitogenicity on mouse thymocytes (------).

cell populations were obtained from peripheral blood, isolated as above and used without further purification.

Supernatants from normal B cells and B cell lines. Peripheral blood samples from normal donors were depleted of T cells by repeated rosetting and Ficoll-Hypaque centrifugations. B cells with <1.0% T cells contamination were cultured at different concentrations, between 2 and 25 × 10^6/mL. Supernatants were collected after 24 hours and tested for direct mitogenic effect in the mouse thymocyte assay. B cell lines were cultured at a concentration of 1 × 10^6/mL, and supernatants were collected at 24 hours.

IL 1 assay and comitogenicity assays. IL 1 activity was measured in the standard mouse thymocyte proliferation assay.[22] In short, 1.5 × 10^6 thymocytes from C3H/HeJ mice were cultured for 72 hours in standard 96-well microplates and pulsed for the last 8-hour period with 1 μCi ^{3}H-thymidine. Supernatants to be tested were diluted twofold from a starting concentration of 50%. These were either tested alone or in the presence of phytohemagglutinin (PHA) (5 μg/mL) or a combination of phorbol ester TPA (30 ng/mL) and calcium ionophore A 23187 (0.1 μmol/mL) for a comitogenic response. All measurements were done in triplicate, and only representative experiments are given in which mean error deviations were <5%.

BCGF, BCDF, and IL 2 assays. BCGF activity was tested on purified and anti-IgM prestimulated normal tonsillar B cells as described.[23] BCDF activity was evaluated by secreted IgG in BCGF assays. IL 2 activity was measured on the IL 2-dependent mouse T cell line CTLL-2 as described.[24]

Radioimmunoassay. A commercial radioimmunoassay for determination of IL 1β was used. In brief, anti IL 1β was mixed with standard or test samples and incubated for 16 hours at room temperature before ^{125}I-IL 1β was added. After an additional 16 hours, antibody-bound–labeled IL 1β was separated by addition of polyethylene glycol 6000 in phosphate-buffered saline (PBS) containing anti-rabbit IgG serum, followed by centrifugation. Supernatants were then decanted and labeled antibody-bound IL 1β was counted in a γ counter (1282 Compugamma LKB). Duplicate controls for total counts (^{125}I-IL 1β alone) and nonspecific binding were also included. Calculations of the bound IL 1β were made according to a standard formula, and interpolations were made from a standard curve.

Preparation of B-CLL–derived IL 1-like factors (B-CLL IL 1). B-CLL were obtained by leukapheresis, washed several times in PBS with 5% FCS, and counted. Contaminating RBCs were removed by centrifugation on Ficoll-Hypaque if necessary. Cells were adjusted to the desired cell concentration in RPMI 1640 medium with antibiotics and 5% FCS and added to 24-well Costar plates in a total volume of 2 mL. Bulk preparations of B-CLL IL 1 were produced by culturing B-CLL at high concentrations (usually 30 × 10^6/mL) in tissue culture bottles. Serum-free preparations of B-CLL IL 1 were produced in adjusted Iscove's medium containing transferrin (12.5 μg/mL), bovine serum albumin (BSA) (400 μg/mL), and 2-mercaptoethanol (5 × 10^{-5} mol/L).

HPLC chromatography. B-CLL from patient AD were cultured for 2 days in Iscove's medium containing 400 μg/mL BSA (Boehringer, Darmstad, FRG) and 12.5 μg/mL human transferrin (Kabi, Stockholm) with penicillin, streptomycin, and L-glutamine as additives. The cells were seeded at 5 × 10^6/mL and kept in a humidified incubator at 37°C in 5% CO$_2$. To determine the biochemical properties and the mol wt of the B-CLL IL 1, released supernatants were concentrated and dialyzed against 10 mmol/L phosphate buffer pH 7.2 containing 1 mmol/L Ca Cl$_2$ on collodium bags with 10-kd mol wt cutoff (Schleicher and Schull, FRG). The concentrated material (2 mL) was purified in a two-step high-performance liquid chromatography (HPLC) procedure according to Köck and Lugger. First, a hydroxylapatite separation on a Biogel HPHT column was performed using a 10 to 500 mmol/L sodium phosphate gradient. The activity peak of IL 1 was concentrated and dialyzed against PBS + 0.1% polyethylene glycol (PEG) 4000 (Merck). The material (0.2 mL) was separated on an Ultro-pac TSK-G2000 SW (7.5 × 600 mm) column equilibrated with PBS + 0.1% PEG 4000, collecting 2-mL fractions (Fig 1). Fractions were tested at dilutions of 1:4 and 1:8 in the mouse thymocyte assay. Mol wt markers were blue dextran (2 × 10^3 kd) like the void volume, and BSA (68 kd), ovoalbumin (45 kd), and RNA (13.7 kd).

Reagents and antisera. PHA was purchased from Wellcome Diagnostics (Dartford, England). Phorbol ester TPA, 2-aminoethyl isotiouronium bromide hydrobromide, and Calcium ionophore A

Table 1. B-CLL–Derived IL 1 Activity in 24-Hour Supernatants

Cultured Cells	T Cell Markers*		Response in Mouse Thymocyte Assay†				
	CD3	RFC	50%‡	25%	12.5% (cpm × 10^3)	6.2%	Control
Unfractionated	2.9	3.1	34.1	30.0	16.5	4.6	0.5
T cell depleted	<0.5	<0.5	25.2	31.2	21.5	11.9	0.4

*Surface markers were tested by membrane fluorescence with anti-CD3 antibodies and with SRBC rosette formation. T cells were depleted by rosette sedimentation.

†Mouse thymocytes were tested for direct mitogenicity in a standard 72-hour IL 1 type assay.

‡Percentage of supernatant in assay.

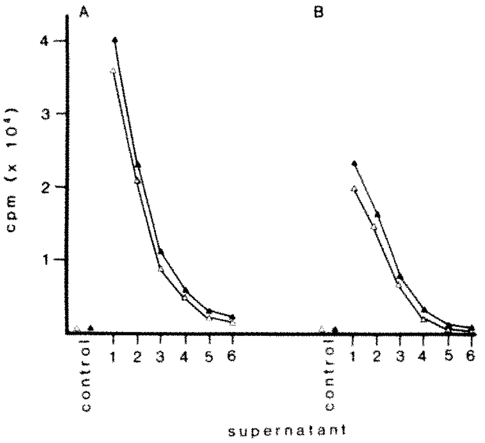

Fig 2. Supernatant from AD B-CLL culture was tested for IL 1-like activity on mouse thymocytes in a standard 72-hour assay. Supernatant was added at different concentrations (1 to 6) starting from 50% and using twofold dilutions. Mouse thymocytes were used at cell concentrations 8 × 10^5/well (A) and 4 × 10^5/well (B) with (△—△) and without (▲—▲) PHA at 5 μg/mL.

23187 were purchased from Sigma Chemical (St Louis). Blue dextran, BSA, ovoalbumin, RNA and concanavalin A (Con A) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Antisera against IL 1, purified IL 1, and IL 1β (^{125}I) radioimmunoassay (RIA) were purchased from Eurogenetics N. V. (Cistron, Biotechnology), Belgium.

RESULTS

B-CLL–derived IL 1-like growth factors in supernatants from tissue-cultured cells. B-CLL were isolated from a patient undergoing regular leukapheresis as part of the treatment schedule. The cells were tissue cultured at a concentration of 4 × 10^6/mL in RPMI 1640 with 5% FCS. Supernatants were analyzed for BCGF, BCDG, or IL 1 and IL 2 activity in standard biologic assays.[22-24] At no time (24, 48, and 72 hours) were any detectable levels of BCGF, BCDF, or IL 2 found (not shown) although an IL 1-like activity was already apparent at 24 hours (Table 1). An even higher factor activity was found if serum-free conditions were used. Among the B-CLL a small contamination of normal T cells was found, but no levels of CD 16 (NK) or CD 13 (monocytes)-positive cells were detectable. If the small fraction of normal T cells was removed by rosetting, similar levels of B-CLL IL 1 activity remained (Table 1).

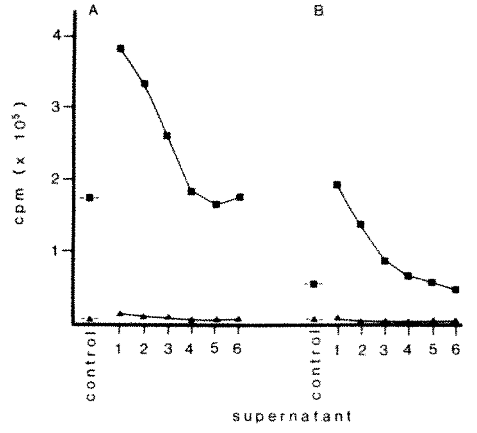

Fig 3. Supernatant from AD B-CLL culture was tested for IL 1-like activity on mouse thymocytes in a standard 72-hour assay. Supernatant was added at different concentrations (1 to 6) starting from 50% and using twofold dilutions. Mouse thymocytes were used at cell concentrations 4 × 10^5/well (A) and 2 × 10^5/well (B) with (■—■) and without (▲—▲) Con A at 5 μg/mL.

Table 2. Comitogenic Effect of B-CLL IL 1

Comitogen	Response in Mouse Thymocyte Assay* 50%†	25%	12.5% (cpm × 10^3)	6.2%	Control
Medium	23.6	18.7	11.5	6.3	0.4
TPA/ionophore‡	359.1	186.4	60.2	10.4	2.1
TPA	0.1	0.4	0.3	0.3	0.3
Ionophore	0.5	0.7	0.6	0.1	0.2

*Comitogenicity in a standard 72-hour IL 1 type assay.

†Percentage of supernatant in assay.

‡Phorbol ester TPA was used at a final concentration of 30 ng/mL and calcium ionophore A 23187 at 0.1 μmol/mL.

Comitogenic properties of B-CLL IL 1. To characterize B-CLL IL 1 functionally, the comitogenic properties were tested with PHA, Con A, and/or combinations of phorbol ester TPA and calcium ionophore A 23187. B-CLL IL 1 was directly mitogenic for mouse thymocytes, and the addition of PHA did not influence the results (Fig 2). With Con A instead of PHA, however, a direct mitogenic response to the lectin occurred that was markedly potentiated by B-CLL IL 1 (Fig 3). In comparison with B-CLL IL 1's direct mitogenicity at a final supernatant concentration of 50%, Con A-dependent comitogenicity gave approximately ten times

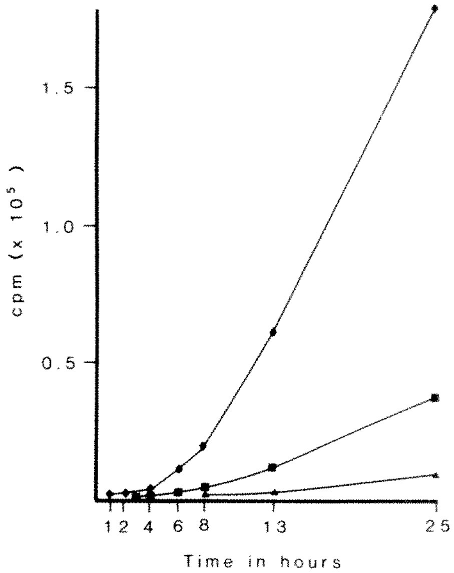

Fig 4. AD B-CLL were cultured at 4 × 10^6/mL. Supernatants were collected at different times and tested at final concentrations of 50% (♦—♦), 25% (■—■), and 12.5% (▲—▲) for a comitogenic response with TPA (30 ng/mL) and calcium ionophore A 23187 (0.1 μmol/mL). Background proliferations were <1 × 10^3 cpm.

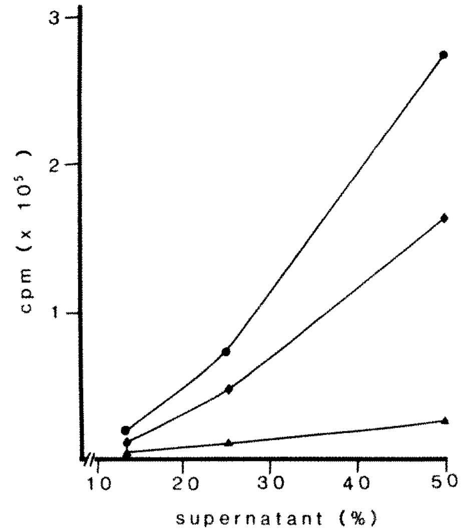

Fig 5. AD B-CLL were cultured at cell concentrations 8 × 10^6/mL (●—●), 4 × 10^6/mL (♦—♦), and 2 × 10^6/mL (▲—▲). Supernatants were collected at 24 hours and tested with calcium ionophore A 23187 and TPA (described in legend to Fig 4) for induction of mouse thymocyte proliferation. Background proliferation was 0.7 × 10^3 cpm.

more counts per minute per cell basis although the background was considerable. To optimize B-CLL IL 1 detection, combinations of TPA and calcium ionophore were tested for comitogenicity. TPA at 30 ng/mL and calcium ionophore A 23187 at 0.1 μmol/mL did not stimulate any background proliferation but allowed a sensitive detection of an IL 1-like comitogenic effect (Table 2).

Kinetics and dose dependency of B-CLL IL 1 formation. B-CLL cells were tissue cultured at 4 × 10^6/mL. Supernatants were collected at different times and tested at final concentrations of 50%, 25%, and 12.5% for a comitogenic response with TPA and calcium ionophore. With 50% supernatants, some B-CLL IL 1 activity was already detectable at 6 hours (Fig 4). Peak activity was obtained at 24 hours (not shown).

A dose-response relationship was established between the number of cultured B-CLL and B-CLL IL 1 activity in the TPA/calcium ionophore coresponse assay (Fig 5). At more than 4 × 10^6 B-CLL/mL there appeared to be a linear correlation, as 8 × 10^6/mL gave almost a doubling of the proliferative response.

Effect of B-CLL IL 1 on autologous cells and allogeneic thymocytes and lymphocytes. B-CLL IL 1 was tested for direct mitogenicity on mouse thymocytes, autologous B-CLL, and allogeneic thymocytes and lymphocytes. Although B-CLL IL 1 was directly mitogenic for mouse thymocytes, none of the other cell types responded in a 72-hour proliferation assay (Table 3). When B-CLL IL 1 was tested for comitogenicity with TPA/calcium ionophore on allogeneic thymocytes and lymphocytes, there was no response of the thymic cells but a clear low-grade response of the peripheral lymphocytes (not shown).

Inhibition of B-CLL IL 1 by anti-IL 1. Two different anti-IL 1 preparations were tested for inhibition of B-CLL IL 1-dependent direct mitogenicity on mouse thymocytes (Table 4). One antisera was an IL 1-neutralizing polyclonal rabbit antiserum; the other was a nonneutralizing monoclonal antibody. Purified IL 1 was also included in the assay. The neutralizing antiserum neutralized both IL 1 as well as B-CLL IL 1 to a similar degree.

Study of B-CLL IL 1 by HPLC chromatography and activity of the fractions. Supernatants were concentrated ten times, purified, and analyzed as described in the Materials and Methods section, after HPLC hydroxylapatite and gel filtration. As shown in Fig 1, the highest IL 1-like activity was related to the 17-kd region, which is in concordance with previously reported mol wt for human IL 1. The effect from B-CLL IL 1 on the mouse thymocyte proliferation assay was directly observed in the absence of PHA or Con A.

B-CLL IL 1 activity in unfractionated leukocyte preparations from different chronic leukemias. Whole leukocyte fractions from five additional chronic leukemic patients were cultured for 24 hours, and supernatants from cultures were tested for IL 1-like activity, measured as direct mitogenicity or comitogenicity with TPA/calcium ionophore on mouse thymocytes (Table 5). All three cases of B-CLL were positive in both systems, with a strong potentiation by the presence of TPA/calcium ionophore. With the other leukemias, a low-grade induction of direct mitogenicity, less amplified by TPA/calcium ionophore as compared with B-CLL, was found. WBC counts varied widely; however, >90% were leukemic cells.

Identification of IL 1β by RIA. IL 1-like activity in supernatants from leukemic, cultured cells was analyzed by means of an IL 1β (^{125}I) RIA. A comparison between the mouse thymocyte assay and IL 1β (^{125}I) RIA was done (Table 6). B cell line supernatants were negative in both

Table 3. Effect of B-CLL IL 1 on Autologous and Allogeneic Cells

		Response in Proliferation Assay*				
Experiment	Cell Population	50%†	25%	12.5% (cpm × 10^3)	6.2%	Control
1	Mouse thymocytes	26.2	15.3	12.5	1.1	0.9
	Autologous B-CLL	0.8	0.7	0.7	0.4	0.3
	Human PBLs	2.2	1.9	2.1	2.4	2.1
2	Mouse thymocytes	18.1	16.5	10.2	6.9	0.4
	Human thymocytes	7.4	8.9	9.0	10.2	9.8

PBL, peripheral blood lymphocytes.

*B-CLL IL 1 was derived from a 24-hour cell culture and tested on the different cells in a 72-hour proliferation assay.

†Percentage of supernatant in assay.

Table 4. Inhibition of B-CLL IL 1 Activity by Anti-IL 1

		Response in Mouse Thymocyte Assay							
		Control†	Medium‡		Poly anti-IL 1		Mono anti-IL 1		
Experiment	Factor		50%§	25%	50% (cpm × 10³)	25%	50%	25%	Inhibition*
1	B CLL IL 1	0.4	12.8	1.8	1.3	0.3	11.4	2.0	89.0%
	IL 1‖	0.4	11.8	1.4	1.8	1.2	13.4	2.2	84.7%
2	B CLL IL 1	0.5	16.0	4.6	4.7	1.3	ND	ND	70.6%

Polyclonal anti-IL 1 was produced in rabbits and used at a final dilution of 1:500. The equivalent 1:500 dilution of normal rabbit serum did not influence IL 1 activity.

*Inhibition at 50% with polyclonal anti-IL 1.

†Background cultures in absence of stimulation.

‡Direct mitogenic effect.

§Percentage of supernatant in assay.

‖Purified monocyte IL 1.

assays. Supernatants from normal cultured B cells were negative in the mouse thymocyte assay. There was no direct correlation between results from the different assays. Supernatants containing high concentrations of IL 1β did not correspond to higher responses in the mouse thymocyte assay. Particularly with the supernatants from patients AD and JÅ no such correlation was found.

DISCUSSION

Isolated B-CLL spontaneously produced IL 1-like factors in vitro but not BCGF, BCDF, or IL 2. Similarly to human monocyte IL 1, B-CLL IL 1 was directly mitogenic for mouse thymocytes and comitogenic with Con A.[25,26] The mouse thymocyte proliferation assay was not influenced by PHA in contrast to the effect of Con A. In absolute numbers of counts per minute, the comitogenicity with Con A gave approximately five to ten times more counts although supernatants titered to similar endpoints in both assays. Because Con A also is directly mitogenic for mouse thymocytes, we used a nonmitogenic combination of phorbol ester TPA and calcium ionophore. With this combination, the concentration of the ionophore (0.1 μmol/mL) was crucial to avoid background stimulation and to optimize comitogenicity.

B-CLL produced strong B-CLL IL 1 activity that was easily detected if tested for comitogenicity with TPA/calcium ionophore. Among the different leukemic cells there was no obvious relationship between disease activity and B-CLL IL 1 production. Only AD cells, however, were tested as purified B-CLL. Thus, normal cells were present among the leukemic cells, although they comprised <10%. These cells may contribute to the spontaneous production of IL 1.[17,25] Hence, a clear B-CLL origin of B-CLL IL 1 was only established with AD cells. This patient died 10 months after initiation of the study. Numerous cells from leukapheresis were obtained in numbers $>10^{11}$ and can thus serve as an abundant source for further isolation and characterization of B-CLL IL 1.

B-CLL IL 1 production in the present system was spontaneous and did not require stimulation with LPS as noted earlier.[17] Although an endotoxin contamination in FCS, present in culture medium, was not formally ruled out, B-CLL IL 1 production was even higher if serum-free conditions were used (data not shown) as described for IL 1. B-CLL IL 1 production did not require proliferation of B-CLL because these were essentially nonproliferative and factors production was detectable as early as after 6-hour culture at high cell concentration as noted earlier.[17] Although B-CLL cells did not proliferate spontaneously, either with endogenous or additional B-CLL IL 1, cells could be triggered to divide at 48 hours with TPA and calcium

Table 5. B-CLL IL 1 Activity in Unfractionated Leukocyte Preparations From Different Chronic Leukemias

					Response in Mouse Thymocyte Assay			
Patients*	Leukemia Type	Progressive Disease	Rai Stage	WBC†	Control‡	SN§ (cpm × 10³)	TPA/Ion‖	Mixture¶
AD	B-CLL	Yes	IV	335	0.4	19.4	2.2	360.1
BF	B-CLL	No	0	21	0.5	8.8	0.9	222.4
GP	B-CLL	Yes	III	23	0.3	4.6	0.3	40.2
MS	CMML	Yes	NA	26	0.4	3.5	0.3	8.2
IU	T	No	0	25	0.7	2.5	0.6	9.4
EL	T	Yes	IV	300	0.6	1.1	0.7	5.3

Chronic myelomonocytic leukemia; NA, not applicable.

*Clinical and laboratory data at test.

†Lymphocytes × 10⁹/L.

‡Background.

§Supernatants from 24-hour cultured cells (without IL 2 activity) were tested at 25% for direct mitogenic effects.

‖TPA 30 ng/mL and calcium ionophore A 23187 at 0.1 μmol/mL.

¶TPA/Ion and SN tested together.

Table 6. B-CLL IL 1 Activity in Comparison to IL 1β Concentration

Patients* and Cell Sources	Leukemia Type	Progressive Disease	Rai Stage	WBC†	Response in Mouse Thymocyte Assay‡			IL 1β (ng/mL)
					Control	Test (cpm × 10^3)	% SN§	
L.K.	B-CLL	No	I	26 (ND)	0.3	3.1 (10)‖	50.0	50.0
H.M.	B-CLL	No	I	42 (ND)	0.3	2.0 (6)	12.5	43.0
G.G.	B-CLL	Yes	IV	73 (4.0)	0.3	6.4 (19)	25.0	21.0
S.I.	B-CLL	Yes	III	10 (ND)	0.3	5.9 (18)	50.0	7.0
H.G.	B-CLL	Yes	I	79 (1.0)	0.3	5.2 (15)	50.0	6.0
G.H.	B-CLL	Yes	IV	26 (3.0)	0.3	6.2 (18)	50.0	1.6
J.Å.	B-CLL	Yes	I	75 (ND)	0.5	1.7 (3)	12.5	1.4
A.D.	B-CLL	Yes	IV	335 (3.1)	0.7	11.0 (16)	50.0	0.5
Raji¶	—	—	—	—	0.7	2.4 (3)	25.0	0.0
Daudi	—	—	—	—	0.7	1.9 (3)	6.2	0.0
BL-28	—	—	—	—	0.3	0.4 (1)	50.0	0.0
Normal 1**	—	—	—	—	0.4	1.6 (4)	50.0	ND
Normal 2	—	—	—	—	0.4	0.8 (2)	50.0	ND
Normal 3	—	—	—	—	0.4	1.4 (3)	6.2	ND

*Clinical and laboratory data at test.

†Number of lymphocytes × 10^9/L (percentage of CD2-positive T cells by fluorescence).

‡Direct mitogenic effect.

§Percentage of supernatants in test from 40 × 10^6 cells/mL cultured for 24 hours.

‖Stimulation index.

¶Supernatants from Burkitt lymphoma-derived cell lines.

**Supernatants from normal peripheral blood B cells (< 1.0% T cells) cultured at 2, 10, and 25 × 10^6/mL by 24 hours.

ionophore (data not shown). Whether endogenous B-CLL IL 1 is important for this proliferation is unclear.

Inhibition of B-CLL IL 1 activity by an IL 1-neutralizing polyclonal rabbit antiserum, but not by a monoclonal antibody, strongly indicated a similarity to monocyte-derived IL 1. Additional neutralization studies with anti-IL 1α and β, should contribute to its further definition. Also the chromatographic behavior of B-CLL IL 1 on HPLC-HPHT (high performance hydroxylapatite) and TSK-G2000 SW columns suggested a similarity to monocyte-derived IL 1. The binding properties on a hydroxylapatite Biogel HPHT column with elution at 0.23 to 0.28 mol/L sodium phosphate are identical for B-CLL IL 1 and monocyte-derived IL 1. TSK-G2000 SW HPLC gel filtration also gives similar activity peaks for B-CLL IL 1 and monocyte IL 1 (10 to 20 kd with maximum at 17 kd). In addition, it is clear that IL 1β was spontaneously produced by the leukemic cells. Supernatants from normal B cells and B cell lines did not produce significant stimulation in the mouse thymocyte assay. B cell lines did not produce detectable levels of IL 1β.

The thymocyte assay and the IL 1β (^{125}I) RIA did not give identical results (Table 6). One supernatant with a high RIA level gave a poor thymocyte proliferative response (HM in Table 6), and several high responders in the thymocyte assay were low in RIA measurements. This indicates production of enhancing/suppressor factors by the B-CLL and/or the formation of different IL 1-like factors.

The biologic and clinical significance of B-CLL IL 1 is still unclear. The detection of IL 1-like factors in nonmonocytic cells is not surprising because both normal B cells and, indeed, most nucleated cells can produce similar factors.[16] A further characterization and analysis of B-CLL IL 1 should be done in accordance with the work by Wakasugi et al,[19,20] who demonstrated a novel type of IL 1 in an EBV-containing B cell line which functions as an autocrine growth factor. The described IL 1 was not neutralized by polyclonal antimonocytic IL 1 antibodies, however, and thus was distinct from our B-CLL-derived IL 1-like factor.

Pistoia et al analyzed B-CLL IL 1 production in purified B-CLL from 12 patients and found activity in nine but not obvious relationship to the stage of the disease.[17] In additional work, IL 1 activity should be measured in serum and urine in individual patients, and an effort should be made to analyze whether increased levels of IL 1 influence T cell immunity in these patients as the T cell system is clearly aberrant in B-CLL.[5,6] Another interesting question is whether increased IL 1 concentrations, either systemic or local, increase stem cell activity in bone marrow for the B-CLL clone as measured by colony formation in vitro.[27]

In summary, some B-CLL spontaneously produce factors in vitro that have characteristics of IL 1. Among them, IL 1β has been identified. A further characterization of B-CLL-derived factors should be facilitated by the availability of large numbers of clonal cells.

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Dermatan Sulphate Induces Plasminogen Activator Release in the Perfused Rat Hindquarters

By Marzia Abbadini, Guang Jin Zhu, Antonio Maggi, Jerta Pangrazzi,
Maria Benedetta Donati, and Luciana Mussoni

Heparin or heparin-like substances have been described to induce the release of plasminogen activator (PA) activity in different animal perfusion models. In this paper we report that Dermatan Sulphate (DS) is able to induce PA activity release in the perfused rat hindquarters. Perfusion of different doses of DS (0.1 to 0.8 mg/mL) stimulates a release of PA activity that is maximum after the initial two minutes of perfusion. The amount of PA activity released rises progressively within a certain concentration range of DS (0.1 to 0.4 mg/mL) and declines thereafter (0.6 to 0.8 mg/mL). The type of PA activity increased during DS perfusion was characterized by SDS-PAGE and fibrin autography as tissue-type PA (t-PA) on the basis of its mol wt (67,000 d) and inhibition by a specific anti t-PA antiserum. This effect might be considered as potentially contributing to the antithrombotic activity of DS, at least at the local level.

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PREVIOUS investigations have shown that heparin and heparin-like substances can interact with proteins of the fibrinolytic system, both in ex vivo and in vitro studies. In particular, Markwardt et al[1] demonstrated that perfusion of heparin and sulphated polyanions enhance PA release, in the isolated pig ear model, in a dose-dependent manner.[1] Moreover, high and low mol wt heparin preparations (HMW, LMW) induced fibrinolytic activity, both in vitro and in vivo in human volunteers.[2] The same investigators have shown that this effect partially depends on the degree of sulphation of the molecule, since a synthetic polysulphate polysaccharide (SP54) was more active than other heparin preparations tested.[2] The antithrombotic effect of HMW and LMW heparins was lost when antifibrinolytic drugs were administered along with the heparins in the rabbit Wessler model.[3] Further studies with SP54 indicated that this substance induced the release of t-PA if perfused in the rabbit ear and that this activity was involved in the drug's antithrombotic action.[4] Moreover in human volunteers SP54 activates the fibrinolytic system after both oral and subcutaneous administration.[5] Recently it has been hypothesized that a direct interaction of heparin or heparin-like substances with plasminogen and t-PA or urokinase (UK) induces an increase in plasmin formation.[6,7]

Dermatan Sulphate (DS), like heparin, has been shown to be a potent antithrombotic agent in different experimental models of venous thrombosis,[8,9] without affecting the coagulation tests. To clarify the possible mechanisms that may contribute to the antithrombotic efficacy of DS, we studied its interaction of this drug with the fibrinolytic system. In particular, we investigated if DS was able to modify the release of plasminogen activator (PA) from the vascular bed using the perfused rat hindquarters model. This model was proposed[10] as a useful tool to study the physiological and pharmacological modulation of PA release.

MATERIALS AND METHODS

Dermatan Sulphate (DS) MF 701, isolated from pig intestinal mucosa, was kindly supplied by Mediolanum Farmaceutici SRL, Milano, Italy. Heparin contamination was <1% as demonstrated by electrophoretic analysis and the in vitro anticoagulant activity was calculated to be around 5 U/mg USP.

Perfusion Model

The experimental model used was the perfused rat hindquarters described by Emeis.[10] Briefly, 200 to 250 g male CD-COBS rats (Charles River, Italy) were anaesthetized with sodium pentobarbital (50 mg/kg), administered intraperitoneally. After isolating both the aorta and vena cava, up to the ileofemoral bifurcation, a ligature was applied around each vessel. A 22 G needle was inserted, without stasis, into the aorta and perfusion was immediately started at a constant flow rate of 10 mL/min. A cannula was inserted into the vena cava to allow perfusate collection. Perfusion of the hindlimb vascular bed was performed using a Tyrode's solution pH 7.4, containing 0.1% BSA (Sigma Chemical Co, St Louis) and oxygenated with 95% O_2 and 5% CO_2, at 37°C. The perfusion pressure on entry was continuously monitored using a transducer and polygraph (Grass Instrument Co, Quincy, MA).

Experimental procedure. Each experiment started with a 30 minute, wash-out period of the rat hindquarters using only Tyrode's solution. After this initial period, the drug under investigation (dissolved in Tyrode's) was perfused for three minutes, followed by a second wash-out with Tyrode's solution. Samples (1 mL) were collected at regular intervals (five to ten minutes) from the cannulated vena cava during wash-out and every minute during drug perfusion. The samples, collected on ice, were centrifuged (12,000 g for 60 seconds) and analyzed for fibrinolytic activity. Each animal was perfused only once.

Fibrinolytic Activity

Fibrin plate assay. PA activity of the samples, collected during the perfusion period, was measured by a fibrin plate method using plasminogen-rich human fibrinogen (Grade L; Kabi, Stockolm).[11]

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Thirty microliters of each sample were placed (in duplicate) on the fibrin film and the diameter of the lysis areas were measured after 18 hours incubation at 37°C. The PA concentration was expressed as PA mU/mL of perfusate in comparison to a reference curve obtained, for each set of experiments, using known concentrations of t-PA standard (WHO, London, coded 831517). The results were analyzed statistically by Duncan's test.[12]

Fibrin autography. The perfusion samples, collected as described in the experimental procedure, were kept at −20°C in 0.01% Tween 80 until tested. The molecular type of PA was evaluated by a fibrin autography technique[13] after SDS-PAGE. The apparent mol wt of PA was calculated by comparing the migration distance of the lysis area with those of marker proteins of known mol wt (Pharmacia, Uppsala, Sweden), run simultaneously. The immunological characterization was performed with home-made rabbit antibodies against human t-PA or UK incorporated in the fibrin agar plate.

RESULTS

Using the rat hindquarters perfusion model, we observed that DS was able to stimulate the release of PA activity in a dose dependent manner (in the range of 0.1 to 0.4 mg/mL). At higher DS concentrations (0.6 to 0.8 mg/mL) there followed a gradual decrease in the lysis of fibrin plates (Fig 1). Throughout the whole of these experiments no increase in basal perfusion pressure (50 to 60 mmHg) was recorded.

If we examine the profile of PA activity measured in the perfusate with the DS dose giving the highest PA activity release (0.4 mg/mL), we can observe (Table 1) that the maximum PA level was achieved during the first initial minutes of perfusion progressively declining thereafter to basal values 50 to 60 minutes from the beginning of the experiment.

Table 1 reports PA activity released from vascular cells during the perfusion of DS at doses of 0.1 to 0.4 mg/mL. The release of PA activity induced by DS (0.2 to 0.4 mg/mL) was maximal and significantly different from baseline levels during the first two minutes of DS perfusion and decreased thereafter. Above 0.4 mg/mL a reduction in PA was also seen, 0.6 mg/mL inducing a similar release of PA as the 0.1 mg/mL dose.

Different heparin preparations, tested at concentrations comparable with DS, were found ineffective (Fig 1); moreover, also lower concentrations of heparin (0.0001 to 0.01 mg/mL) were devoid of activity (data not shown).

The samples collected from the vena cava both during Tyrode's and DS perfusion were examined by fibrin autography after SDS-PAGE to determine the type of PA released. The perfusate contained two types of PA, which appeared on a fibrin-agar plate as lysis bands of 67,000 and 43,000 mol wt (Fig 2). It can clearly be seen, that both in control samples (collected during the first wash-out period) and in the stimulated samples (collected in the first minute of DS perfusion), both bands appeared and that perfusion of DS caused an increase only in the band corresponding to a 67,000 mol wt while the lower mol wt band was unaltered. The 67,000 mol wt band is blocked by an anti–PA antiserum and the 43,000 mol wt band partially inhibited by an anti-human UK antibody, as previously described.[14]

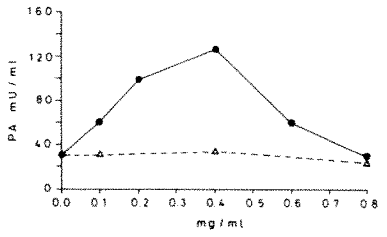

Fig 1. PA activity released as a function of DS (●) and heparin (△) concentrations perfused. The data reported here refer to the first minute of perfusion. Each value is the mean of five experiments performed on different animals (SD never exceeded 10% of the mean).

Table 1. PA Activity Released in Rat Hindquarters Perfused With Different DS Concentrations

Perfusion Time (min)		PA Released (mU/mL) After DS Perfusion*		
		0.1 mg/mL n = 5	0.2 mg/mL n = 7	0.4 mg/mL n = 9
25	Tyrode's	33 ± 3.2	36 ± 3	34 ± 4
30	DS	60 ± 13	99 ± 23†	127 ± 32†
31	DS	61 ± 20	97 ± 32†	129 ± 33†
32	DS	57 ± 17	75 ± 18†	89 ± 19†
35	Tyrode's	53 ± 10	59 ± 10	72 ± 12
40	Tyrode's	44 ± 9	63 ± 9	62 ± 10
50	Tyrode's	37 ± 4	59 ± 8	44 ± 6
60	Tyrode's	35 ± 4	55 ± 6	46 ± 7

Note. n, numbers of animals; mg/ml, dose of DS.

*Mean ± SEM.

†$P < .01$ Duncan's test (*v* Tyrode's sample collected after 25 minutes).

To verify whether DS had a direct fibrinolytic activity on fibrin films or an in vitro interaction with PA molecules, DS

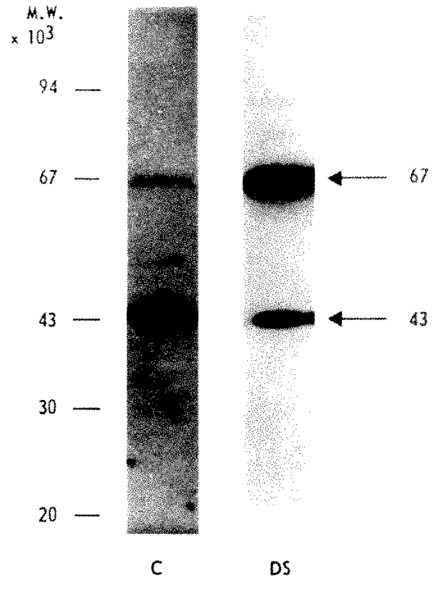

Fig 2. Fibrin autography of samples from perfused rat hindquarters. Line (C) corresponds to the perfusate collected after 25 minute wash-out with Tyrode's solution alone and line (DS) to the sample collected after the first minute of perfusion with 0.4 mg/mL of the drug. The arrows indicate the apparent mol wt corresponding to the lysis area.

(0.1 to 0.8 mg/mL) was dissolved in Tyrode's solution and placed directly on a fibrin plate, alone or together with different concentrations (10 to 100 mU/mL) of standard t-PA. DS alone did not lyse and did not potentiate the activity of standard t-PA. Moreover, no increased activity was observed when Tyrode's perfusion fluid was used as a source of PA instead of t-PA standard (data not shown).

DISCUSSION

DS is one of the components of the extracellular matrix; it is found alone or in association with other glycosaminoglycans in human coronaries, in bovine and Rhesus monkey aorta.[15,16] Unlike heparin, DS inhibits thrombin formation by interacting with only heparin cofactor II and not with antithrombin III.[17,18] Recently, it was shown that DS displays antithrombotic activity in models of experimental venous thrombosis.[8,9] In contrast to heparin, the antithrombotic activity was achieved at doses devoid of hemorrhagic effects.[9] These characteristics make DS a potentially interesting molecule for thrombosis prevention and call attention to the possible mechanisms of its peculiar antithrombotic effect. This paper reports the original observation that perfusion of DS induces an increase in fibrinolytic activity in the rat hindquarters model. The increase was dose-dependent, in a range of 0.1 to 0.4 mg/mL, but decreased following a further increase in the doses used. The rise in fibrinolytic activity was concomitant with the presence of DS in the microvasculature; maximum release was achieved during the initial minutes of perfusion followed by a slight decrease during the subsequent minutes (Table 1). The fibrinolytic activity induced by DS has been identified as t-PA–like molecules on the basis of the relative mobility in SDS-PAGE followed by fibrin autography (Fig 2). We have previously shown[14] that anti–PA antiserum could block the lytic activity of this molecule. This effect was peculiar for DS; indeed, in the same experimental conditions and at the same and lower dosages, different heparin preparations did not induce PA activity release. An in vitro effect of DS on plasminogen activation by t-PA and UK, recently described for heparin and DS,[6,7] was excluded by incubating both the standard t-PA preparation and Tyrode's perfusion fluid (as a source of PA activity) with DS (0.1 to 0.8 mg/mL) and measuring the fibrinolytic activity on fibrin films. We can thus conclude that a direct effect of DS on the vascular wall induces the release of t-PA–like molecules presumably from the endothelial cells.

On the basis of these data, we can thus put forward the hypothesis that part of the antithrombotic activity shown by DS[8,9] in experimental animals, might be due to its direct effect on endothelial cells. The PA released during intravenous (IV) administration of DS in the vicinity of the thrombus could thus bind to fibrin and express its thrombolytic action. On the other hand, the location of naturally occurring DS in subendothelial layers[15,16] could further contribute to the stimulation of PA release from surrounding endothelial cells in cases of vascular injury. Further in vivo studies are required to substantiate the role of endogenous and exogenous DS in thrombosis control.

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Deoxygenation Inhibits the Volume-Stimulated, Cl^--Dependent K^+ Efflux in SS and Young AA Cells: A Cytosolic Mg^{2+} Modulation

By Mitzy Canessa, Mary E. Fabry, and Ronald L. Nagel

We recently reported that the Cl^--dependent K^+ (K:Cl) efflux, which can be stimulated by cell swelling in the presence of inhibitors of the Na^+ pump (ouabain) and of the Na-K-Cl cotransport (bumetanide), is highly active in young AA and SS RBCs. We report here that deoxygenation inhibits volume-stimulated K:Cl efflux in SS and reticulocyte-enriched density-separated SS and AA RBCs. In SS whole blood, the K:Cl efflux stimulated by hypotonic (220 mOsm) swelling was reduced from 9.2 ± 2 (mean ± SE) in oxygen to 2.7 ± 1.9 (mmol/L cell/h – flux units = FU) (n = 4) under deoxygenated conditions ($P < .005$). Deoxygenation also decreased the acid pH-stimulated K:Cl efflux from 5.9 ± 1.5 to 3.7 ± 1.1 FU (n = 3) ($P < .025$) but did not inhibit NEM-stimulated K:Cl transport. The effect of deoxygenation on density-separated SS cells is similar: When fraction SS2 (reversible discocytes) is deoxygenated under hypotonic conditions, the K:Cl efflux is reduced by 50%. In reticulocyte-enriched AA cells obtained from anemic patients, deoxygenation under hypotonic conditions also reduces K^+ efflux by 50%. In SS cells only, deoxygenation under isotonic conditions results in an increased Cl^--independent K^+ efflux. Because ionized Mg^{2+} in the cytosol increases during deoxygenation, we investigated the effect of external and internal Mg^{2+} on the volume-stimulated K:Cl efflux. Removal of external Mg^{2+} did not influence the rate of transport in oxygenated cells. When internal Mg^{2+} was clamped at 0.15 mmol/L with A23187 and EDTA at ionized cytosolic Ca^{2+} = 0, however, the inhibitory effect of deoxygenation on the K:Cl efflux was eliminated. We conclude that deoxygenation inhibits the volume-stimulated, Cl^--dependent K^+ efflux in AA and SS young red cells by concomitantly increasing ionized cytosolic Mg^{2+}.

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WE RECENTLY reported the existence of a Cl^--dependent K^+ (K:Cl) efflux in SS, SC, and CC cells that is involved in volume reduction secondary to cell swelling.[1] This pathway of K^+ transport is insensitive to ouabain and bumetanide and is stimulated by cell swelling induced by hypotonic and acid pH medium containing Cl^- but not NO_3.[1] Another important property of the K:Cl transport system is that it is stimulated by *N*-ethylmaleimide (NEM). The K:Cl efflux stimulated by cell swelling and NEM was elevated to a variable extent in HbS when compared with mature AA cells. The magnitude of this K^+ efflux varies from patient to patient with sickle cell anemia,[1,2] analogous to the heterogeneity in RBC density and volume distribution previously described.

More recently, we reported[2,3] that this K:Cl efflux is a characteristic of the young AA and SS RBCs; most cells in the blood of a sickle cell anemia patient are young cells due to the very short lifetime of SS cells.[4] Two important questions remain to be answered: the effect of deoxygenation on these fluxes, since it is deoxygenation that elicits the pathophysiologic responses of SS cells; and the relation of the K:Cl efflux to the deoxygenation-induced K^+ efflux first observed by Tosteson et al[5] in sickle cells. We report here that deoxygenation inhibits volume-stimulated K:Cl efflux and that this effect is mediated by cytosolic Mg^{2+}.

MATERIALS AND METHODS

Patient material. Normal controls (homozygous for hemoglobin A) and sickle cell anemia patients (homozygous for hemoglobin S), as ascertained by two types of electrophoresis, donated blood after informed consent at the Heredity Clinic of the Bronx Municipal Hospital. Reticulocyte-enriched blood was obtained from AA individuals recently treated for nutritional anemias. Blood was obtained in heparinized tubes and shipped on ice to Boston overnight under conditions previously described.[1] All experiments were performed within 24 hours of venipuncture.

Measurement of K^+ efflux. Since SS and AA cells contain variable amounts of Na^+ and K^+, all cells and fractions were loaded by a modified nystatin procedure[6] to have 11.5 ± 2 Na^+ and 95 ± 9 K^+ mmol/L cell. K^+ fluxes were determined as previously described[1,3] incubating cells at 2% (vol/vol) hematocrit in the following media (in mmol/L): A, 140 NaCl, pH 7.4; B, 140 $NaNO_3$, pH 7.4; C, 100 NaCl, pH 7.4; D, 100 $NaNO_3$, pH 7.4; E, 140 NaCl, pH 7, F, 140 $NaNO_3$, pH 7.0. All media contained 0.1 ouabain, 1.0 $MgCl_2$ (if not otherwise indicated), 0.1 bumetanide, 10 glucose, and Tris-MOPS of the pH indicated at 37 °C.

Cl^--dependent K^+ transport was estimated by subtracting the flux in isotonic nitrate media from that in isotonic chloride media (A – B), or hypotonic media (C – D), or acid pH media (F – E). The volume-dependent K^+ efflux was estimated from the differences between hypotonic and isotonic NaCl (C – A) medium. The Cl^- and volume dependent K^+ efflux was estimated from the difference between Cl^- and NO_3^- media (C – D). The NEM-stimulated K^+ efflux was estimated adding 1 mmol/L NEM to mediums A, B, C, and D. NEM-stimulated, Cl^--dependent K^+ efflux was estimated from the differences in K^+ efflux in chloride (A – B) and NO_3 (B – D) media. Time-course measurements of K^+ efflux every 10 minutes for 80 minutes indicated that initial rates were held for 30 minutes. For experiments under deoxy conditions, described below, NEM was added prior to deoxygenation.

To study the effect of ionized Mg^{2+} on K^+ efflux, the external Mg^{2+} concentration was varied between 0 and 0.5 mmol/L in the presence and in the absence of 1 mmol/L Tris EDTA pH 7.4 and 60 μmol/L/cells of A23187 as previously described.[7] In two separate experiments, ionized Ca^{2+} was buffered at zero and at 0.137 μ molar by use of 1.0 mmol/L EGTA and A23187, whereas Mg^{2+} was varied

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between 0 and 0.5 mmol/L. Total Mg and Ca were determined by atomic absorption, and ionized Ca^{2+} was determined by a Ca^{2+} electrode.

Deoxygenation of RBCs for flux studies. For the purpose of deoxygenation, the chilled RBC suspensions were distributed in six 15-cc tubes for each flux medium. The rack, placed in a glove bag and on an ice bath, was evacuated and then flushed with nitrogen continuously and the tubes were agitated by hand for 1 minute/tube and were then capped with serum caps. A sample was removed by syringe and read on a Co-oximeter, and three tubes of each set of six were removed for a zero-time measurement of extracellular potassium. The remaining three tubes were then placed in a 37 °C water bath for 30 minutes; at the end of that time, the reaction was stopped by cooling in an ice bath for 2 minutes and then the whole tube was spun for 5 minutes at 6,000 g in a centrifuge at 5 °C. The supernatant was removed with a plastic pipette and transferred to a plastic tube for analysis by atomic absorption spectrophotometry. The final percentage deoxyhemoglobin was determined with a Co-oximeter (Instrumentation Laboratories, Sudbury, MA).

Density gradients. Percoll (colloidal silica coated with polyvinylpyrrolidone: Pharmacia Fine Chemicals, Piscataway, NJ) and Stractan (arabino-galactan polysaccharide, St Regis, West Nyack, NY) (prepared as described by Corash et al)[8] gradients were formed by centrifugation from a mixture of Percoll and Stractan as previously described.[9] Fraction SS2, the discocyte fraction with MCHCs between 33 and 37 g/dL, as previously described,[10] was aspirated by pipette after SS1 was removed. Fraction AA1, which is defined as cells with a MCHC <33 g/dL was similarly isolated. That this procedure results in a narrow range of densities can be seen from the density gradients shown in Fig 1.

If RBCs were to be exposed to deoxygenated conditions on density gradients, they were first deoxygenated by alternately flushing with humidified nitrogen and application of a moderate vacuum. Liganded controls were generated by equilibrating an aliquot of the cell suspension with carbon monoxide (CO). All samples were then incubated at 37°C for 30 minutes under the described conditions in the presence of 0.1 mmol/L ouabain and 10 mmol/L glucose. An aliquot was used to measure pH; another was used to measure percentage of deoxy hemoglobin with a CO-oximeter. A further aliquot of the cell suspension was then transferred in a nitrogen-filled glove bag to a centrifuge tube with 5.9 mL appropriate density gradient mix to which 50 µL 100 mmol/L sodium dithionite stock solution prepared under nitrogen had been added. The tubes with gradient mix and cell suspensions were then centrifuged as usual.

RESULTS

Effect of deoxygenation on K:Cl efflux stimulated by swelling in SS whole blood. Deoxygenation stimulates K^+ efflux from SS RBCs;[5] furthermore, we recently demonstrated that conditions that induce swelling also induce a Cl^--dependent potassium (K:Cl) efflux from SS whole blood (as well as young AA RBCs) under fully oxygenated conditions.[2,3] The following experiments deal with the question of the effect of deoxygenation on the portion of the K^+ efflux that is Cl^--dependent.

Under oxygenated and isotonic conditions, K^+ efflux is not Cl^--dependent (Fig 2). In whole blood of an individual with Hb SS, exposure to hypotonic oxygenated NaCl medium containing ouabain and bumetanide induced "volume-stimulated" increase in the K^+ efflux from 4.0 to 11.8 mmol/

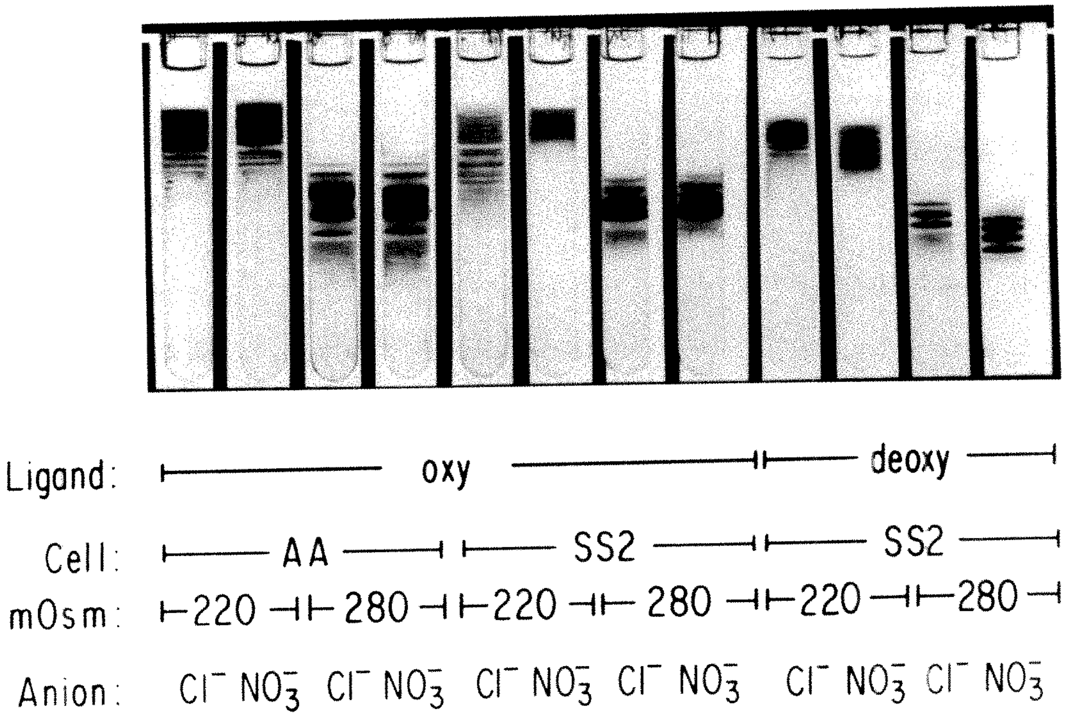

Fig 1. Effect of deoxygenation and hypotonic media containing Cl or NO_3^- on the density distribution of SS discocytes (SS2) and AA whole blood. Percoll-Stractan continuous density gradients were formulated with $C1^-$ or NO_3^- at 220 or 280 mOsm and centrifuged at 37°C. All tubes were at pH 7.4 and had 0.1 mmol/L ouabain. All were preincubated for 30 minutes at 37°C with 10 mmol/L glucose, 10 mmol/L hepes, 0.1 mmol/L ouabain, and osmolarity, anion, and ligand as described in the text. Oxy SS2 cells in hypotonic NO_3^- media have the same density as AA cells in hypotonic media for both the oxy and deoxy states. In contrast, in hypotonic Cl^- media, SS2 cells have a higher density (smaller decrease in MCHC) than do AA cells; however, deoxy SS2 cells have the same density in both Cl^- and NO_3^- media.

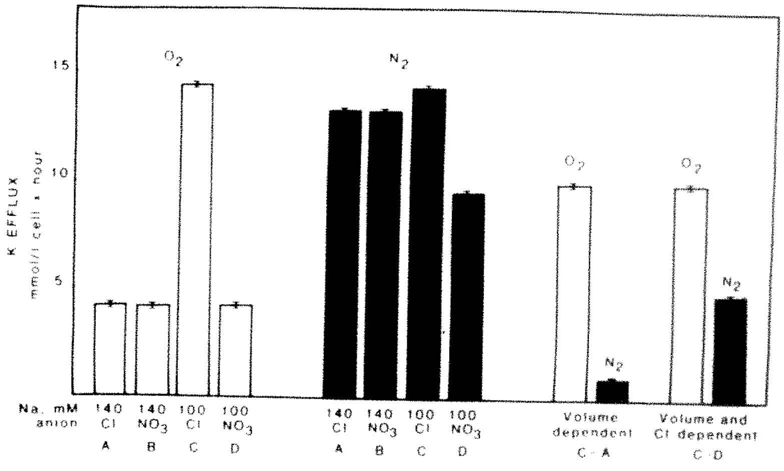

Fig 2. Effect of deoxygenation on initial rates of volume and Cl$^-$-dependent K$^+$ efflux from HbSS RBCs of a single individual. The volume and Cl$^-$-dependent K$^+$ efflux (B through C) is estimated from the differences between hypotonic chloride media (100 mmol/L NaCl = B) and hypotonic nitrate medium (100 mmol/L NaNO$_3$ = C). The volume-dependent K$^+$ efflux from the differences between (C through A) media. RBCs containing 95 mmol/L cell K$^+$ prepared as indicated in the Materials and Methods section were incubated at 2% (vol/vol) hematocrit in NaCl, or NaNO$_3$ as indicated. All media contained (in mmol/L): 1 MgCl$_2$, 10 glucose, 10 Tris-MOPS pH 7.4 at 37°C, 0.1 ouabain, and 0.1 bumetanide.

L/cell × hour (abbreviated FU for flux units) (Fig 2, columns A and C). No stimulation was observed in hypotonic nitrate medium (column D), as expected for a Cl$^-$-dependent phenomena, demonstrating that almost all the volume-stimulated (Fig 2, column C-A) K$^+$ efflux is Cl$^-$-dependent (Fig 2, column D-C). Under isotonic conditions, the increase in K$^+$ efflux produced by deoxygenation clearly is not Cl$^-$-dependent, since it also takes place in nitrate (medium B) medium (Fig 2, A and B dark columns). Deoxygenation did not further increase K$^+$ efflux into hypotonic medium; however, there is still a volume-stimulated, chloride-dependent (Fig 2, C-D) K$^+$ efflux under deoxygenated conditions that is smaller than in oxygenated media. The nearly constant flux into Cl$^-$ media under deoxy isotonic and deoxy hypotonic conditions is due to the large Cl$^-$-*in*dependent K$^+$ efflux into deoxy isotonic media. This can be deduced from the magnitude of K$^+$ efflux-flux into isotonic nitrate medium, which increases from 4.3 (Fig 2, B) to 14.0 (Fig 2, B dark) FU under deoxy conditions (Fig 2) (dark).

Table 1 presents the mean values for the volume-stimulated and Cl$^-$-dependent K$^+$ efflux for four patients with HbSS disease. This K:Cl efflux under deoxygenation was 30% of the flux in oxygen; hence, there was a 70% inhibition (Table 1), and the mean increase of K$^+$ efflux into isotonic nitrate medium in these four subjects was 9 ± .8 FU (data not shown). The negative value of volume-dependent K$^+$ efflux in column B is the result of high values in deoxygenated isotonic chloride media.

We previously showed that cell swelling induced by acid pH 7.0 in isotonic media[1] also stimulates K:Cl efflux. The effect of deoxygenation on the K:Cl efflux stimulated by pH 7 was studied in whole cells from three patients with Hb SS (bottom of Table 1). We found that the volume-stimulated, Cl$^-$-dependent K:Cl efflux decreased 62% upon deoxygenation, whereas the K$^+$ efflux into isotonic NaNO$_3$ medium at pH 7 increased from 4.3 to 13 FU (data not shown).

Previous studies have shown that the K:Cl efflux from HbSS RBCs can also be stimulated by NEM under isotonic conditions.[1] Table 1 also shows that the basal NEM-stimulated K:Cl efflux from whole HbSS RBCs is not significantly different in oxy and deoxy conditions.

Effect of deoxygenation on the volume-stimulated potassium efflux from density-separated SS cells. Since we previously reported that the activity of the volume-stimulated K:Cl efflux is found mostly in young RBCs,[2,3] we fractionated Hb SS RBCs by centrifugation in Stractan-

Table 1. Inhibition by Deoxygenation of Volume and C1$^-$-Dependent K$^+$ Efflux From Whole Blood HbSS RBCs

	K$^+$ Efflux, mmol/L/Cell/h (mean ± SE)			
	A Oxygen	B Nitrogen	A-B Deoxy	*P* Values
A. Hypotonic swelling (n = 4)				
Cl$^-$-dependent*	9.2 (±2.0)	2.7 (±1.9)	6.4 (±0.76)	<0.005
Volume-dependent†	8.6 (±1.5)	−2.4 (±2.7)	10.9 (±2.5)	<0.005
B. Acid pH swelling (n = 3)				
Cl$^-$-dependent‡	5.9 (±1.5)	3.7 (±1.1)	2.2 (±0.75)	<0.025
Volume-dependent§	5.3 (±0.6)	2.0 (±1.1)	3.3 (±0.8)	<0.01
C. NEM-stimulated (n = 4)				
Cl-dependent	34.5 (±0.4)	34.9 (±0.4)	0	NS

BCs were loaded by nystatin to contain K$^+$ 90 m/L/cell. Efflux media contained mmol/L): 0.1 ouabain, 0.05 bumetanide, 1 MgCl$_2$, and 10 Tris-MOPS.

*Cl$^-$-dependent: K$^+$ efflux estimated as the difference between 100 mmol/L Cl$^-$ and 100 mmol/L nitrate medium.

†Volume-dependent: K$^+$ efflux estimated as the difference between hypotonic (100 mmol/L) and isotonic (140 mmol/L) NaCl medium.

‡Cl$^-$-dependent: K$^+$ efflux estimated as the difference between isotonic NaCl, pH 7 medium and isotonic NaCl pH 7.4 medium.

§Volume-dependent: K$^+$ efflux estimated as the difference between pH 7 and 7.4 NaCl (140 mmol/L) medium.

1 mmol/L NEM was used as an activator as described in the Materials and Methods section.

Percoll isopycnic continuous gradients to obtain SS2 containing mainly discocytes. Due to increased RBC destruction, these cells are quite young in SS patients. Table 2 (section A) shows the effect of deoxygenation on basal and on volume-stimulated K:Cl efflux in fraction SS2 RBCs from five subjects with SS disease. The volume-stimulated K^+ efflux under oxygenated conditions varied between 35.9 and 6 FU, whereas efflux from deoxygenated cells varied between 22.4 and 0.8 FU. The volume-stimulated K:Cl efflux decreased by a mean value of 8.4 FU, which represented a 48% inhibition of the values recorded under oxygenated conditions.

Effect of deoxygenation on K^+ efflux stimulated by swelling in AA cells. Although in subjects with SS disease a large fraction of the circulating cells are young cells, in subjects with Hb AA the proportion of reticulocytes is very small (1% to 2%). We have shown, however, that in subjects with recently treated nutritional anemias and a high percentage of circulating reticulocytes, the K:Cl efflux can be as high as that observed in young SS RBCs.[2,3] We report here the effect of deoxygenation on the volume-stimulated K:Cl efflux in reticulocytes (fraction AA1) of three normal subjects (Table 2, section B). As previously reported, all volume-stimulated K^+ efflux was Cl^--dependent in AA reticulocytes under oxygenated and hypotonic conditions. Deoxygenation decreased the magnitude of the volume-stimulated K:Cl efflux from 20.7 to 9.5 FU, which is a 54% inhibition relative to its activity under oxygenated conditions. In AA cells, the volume-stimulated K:Cl efflux is inhibited by deoxygenation without a significant increase in K^+ efflux into nitrate medium as is observed in the RBCs of patients with HbSS (data not shown).

Effect of deoxygenation on the density of SS RBCs in hypotonic medium. Density gradients formulated from Percoll and Stractan are linear for ~ 70% of their span.[11] We have previously demonstrated that when oxygenated SS cells are exposed to hypotonic medium with nitrate as the external anion, they swell, thereby reducing their density, and achieve a final density comparable to that of AA cells under the same conditions.[2,3] In contrast, in Cl^--containing medium, the response of some (30% of SS2, ref. 3), but not all SS cells to hypotonic medium is smaller than that of AA cells. This is due to activation of the volume-stimulated K:Cl efflux in the SS cells, which is accompanied by water loss and in turn results in a lower RBC volume (higher density) at equilibrium. One possible explanation for the inhibitory effect of deoxygenation on the volume-stimulated K^+ efflux is that red cells swell less in hypotonic media under deoxygenated conditions. To test this possibility, we examined AA whole blood and SS2 (density-separated SS discocytes) under oxy and deoxy conditions in isotonic and hypotonic media containing either Cl^- or nitrate as the anion (Fig 1). We found that SS2 cells in nitrate medium respond to hypotonic conditions with a volume increase under both oxy and deoxy conditions. Some,[3] but not all, oxygenated SS cells in hypotonic Cl^- media partially restore their MCHC (by a Cl^--dependent K^+ efflux) to isotonic values, however, and therefore exhibit higher density than cells under the same conditions in nitrate media. In contrast, under deoxy conditions SS2 cells in hypotonic chloride media do not increase their density. This indicates that the volume regulatory decrease response does occur in SS cells under oxygenated conditions and that it is inhibited under deoxygenated conditions.

Effect of Mg^{2+} on K^+ efflux. To study the mechanism of the deoxygenation-induced inhibition of the volume-stimulated K:Cl efflux, we studied the regulatory effect of cytosolic Mg^{2+}. Studies by Flatman and Lew[12] of Mg^{2+} buffering in human RBCs have indicated that at external Mg^{2+} concentration of 0.15 mmol/L (corresponding to our internal ionized concentration of 0.4 mmol/L), the addition of the ionophore A23187 does not produce changes in the Mg^{2+} content of the cells. As shown in Table 3, reduction of external Mg^{2+} did not modify the inhibitory effect of deoxygenation on K:Cl efflux in SS cells. In contrast, when the intracellular Mg^{2+} concentration is fixed at a level between 0 and 0.15 mmol/L with use of the ionophore A23187 and EDTA, deoxygenation no longer inhibited the K:Cl efflux. Furthermore, when the intracellular concentration was raised to 0.5 mmol/L the K:Cl efflux was inhibited even in the oxygenated state.

Because cytosolic calcium is an activator of some K^+ channels, experiments were performed with ionized cytosolic Ca^{2+} equal to zero. To exclude the possibility that it is the removal of cytosolic Ca^{2+} which inhibits the volume-dependent K:Cl transport under deoxy conditions, we also performed two experiments using A23187 + EGTA and varying cytosolic Mg^{2+} while ionized Ca^{2+} was buffered at 0 and

Table 2. Inhibition by Deoxygenation of Volume and Cl^--Dependent K^+ Efflux From HbS Discocytes (SS2) and HbA Reticulocytes (AA1)

	K+ Efflux, mmol/L cell/h (mean ± SE)						
	Basal*		Volume-stimulated				
	Oxygen	Nitrogen	Oxygen		Nitrogen		Δ Deoxy
	Δ Cl	Δ Cl	Δ Cl	Δ V	Δ Cl	Δ V	Δ Cl
A. Hb S discocytes (SS2), n = 5	2.5 (± 1.4)	1.2 (±0.6)	17.3 (±6)	16.4 (±4.5)	8.9 (±4.9)	5.6 (±4.5)	−8.3 (± 1.3)†
B. Hb A reticulocytes (AA1), n = 3	1.6 (± 1.2)	0	20.7 (±8.7)	19.8 (±6.9)	9.5 (±3.2)	9.4 (±3.3)	−10.3 (±3.5)‡

AA1, the least dense AA cells that have an MCHC of <33 g/dL. SS2, discocyte fraction from SS whole blood with MCHCs ranging from 33 to 37 g/dL. Both reticulocytes and dense cells were selectively removed from this fraction. Cl, Cl^--dependent K^+ efflux as defined in Table 1; V, volume-dependent K^+ efflux as defined in Table 1.

*Basal K^+ efflux was measured incubating the K^+-loaded cells (in mmol/L) 140 Na, 0.1 ouabain, 0.01 bumetanide, 10 glucose, and 10 Tris-MOPS, pH 7.4.

†P values <0.005; ‡P values < 0.025.

Table 3. Effect of Ionized Mg^2 on Volume-Stimulated, Chloride-Dependent K^+ Efflux Under Oxygenated and Deoxygenated Conditions from HbSS RBCs

	Medium			A	B	
				Volume-Stimulated–C1$^-$ Dependent K^+ Efflux		
	Mg	A23187	EDTA	Oxygen mmol/l cell/h	N_2	Deoxy (A-B)
1. Tn-575	—	–	+	15.7	11.3	4.4
	—	+	+	10.5	10.3	None
	0.15	+	–	7.6	8.3	None
	0.5	+	–	12.1	6.4	5.7
2. EM-574	—	–	+	5.3	4.0	1.3
	—	+	+	3.7	4.3	None
	0.15	+	–	4.0	4.2	None
	0.5	+	–	5.4	2.5	2.9
3. WR-574	—	–	+	26.3	17.7	8.6
	—	+	+	20	16.0	−4.0
	0.15	+	–	17.5	16.0	1.5
	0.5	+	–	18.7	19.5	None

Incubation contained (in mmol/L) 100 NaCl, or 100 $NaNO_3$ 10 Tris-MOPS (pH 7.4) 60 mmol/L/cell of A21387, 1 mmol/L Tris-EDTA, 10 glucose, 0.1 ouabain, 0.1 bumetanide. The difference between Cl^- and NO_3^- media estimated the volume and chloride-dependent K^+ efflux. The standard deviation of the flux fluctuated between 0.2-0.3 units.

at 0.137 μmol/L, which is somewhat higher than the physiologic cytosolic Ca^{2+} levels of 0.04 μmol/L. These experiments indicate that the removal of Ca did not modify the response of the K:Cl system to deoxygenation.

DISCUSSION

The data demonstrate that the volume-stimulated, Cl^--dependent K^+ efflux (K:Cl efflux) characteristic of SS and young AA red cells[1-3,13] is drastically inhibited by the deoxygenation of these cells. The inhibition of the volume-stimulated K:Cl efflux in both AA and SS cells strongly argues against a specific effect of HbS in the regulation of this pathway. In SS cells only, deoxygenation stimulated a chloride- and volume-insensitive K^+ efflux. The data clearly establish that under hypotonic conditions the deoxygenation-induced K^+ efflux described by Tosteson and colleagues[5] takes place through two pathways which are separate and distinct and, within the conditions studied, noninteractive pathways. This aspect of our findings agree with the observation of Berkowitz and Orringer[14] that deoxygenation in isotonic media stimulates a Cl^--independent K^+ influx.

The study of the inhibition of the volume-stimulated K:Cl efflux by deoxygenation was extended to density-separated RBCs prepared from SS and AA blood. In SS blood, the reversible discocyte fraction with normal MCHC (SS2), which is largely composed of young cells due to the active cell destruction observed in these patients, exhibits approximately double the volume-stimulated K:Cl efflux when compared with whole SS blood; but the inhibitory effect of deoxygenation is similar (~ 50%). The same inhibitory effect is noted in the lightest fraction of AA RBCs (AA1), which contains reticulocytes and other normal RBCs. These results are in accordance with the K:Cl efflux residing largely in the population of young RBCs.

In contrast, the NEM-stimulated K:Cl efflux was not inhibited by deoxygenation. This difference is not surprising because K:Cl transport is also insensitive to ATP depletion whereas the NEM-stimulated flux is inhibited by it,[15] suggesting that different mechanisms of activation are involved.

Studies of the effect of divalent ions on the K:Cl transport in sheep RBCs have shown that high intracellular concentrations of Mg^{2+} inhibit this ion pathway.[15] For this reason, we explored the possibility that cytosolic Mg^{2+} concentrations could mediate the deoxygenation-induced inhibition of the K:Cl efflux. Although removal of external Mg^{2+} did not influence the K^+ efflux in oxygenated or deoxygenated RBCs, clamping of the internal Mg^{2+} by A23187 at 0 to 0.15 mmol/L drastically reduced the inhibitory effect of deoxygenation on the K:Cl efflux. Experiments under oxy and deoxy conditions with both zero and elevated cytosolic ionized Ca^{2+} indicated that Ca^{2+} was not involved in the response of the K:Cl system to deoxygenation. Higher cytosolic concentrations of Mg^{2+} inhibited K:Cl efflux both in oxy and deoxy conditions. Of interest are the recent studies by Bookchin et al[16] indicating that HbSS RBCs have a total Mg^{2+} content similar to that of AA cells.

The increase of free Mg^{2+} in deoxygenated RBCs is principally due to the elevated association constants of deoxygenated hemoglobin with ATP and 2,3-DPG as compared with Mg^{2+}-ATP and Mg^{2+}-2,3-DPG; hence, deoxygenation results in the displacement of Mg^{2+} from these complexes. Evidence that these are the dominant interactions in RBCs comes from the close agreement between the calculated values predicted by Berger et al[17] and the values observed in the intact RBC by P-31 NMR by Gupta et al.[18]

We conclude that the Cl^--dependent K^+ efflux characteristic of young AA and SS cells is inhibited by deoxygenation through the increase in the cytosolic concentration of Mg^{2+} that occurs secondary to the loss of ligand by hemoglobin and the binding to 2,3 DPG.

The presence of a deoxygenation-induced inhibition of K:Cl efflux has potential pathophysiologic importance in sickle cell anemia. Low pH can produce cell swelling and stimulate potassium efflux via this mechanism, which would tend to decrease cell volume. This is a prosickling event as previously discussed[1-3] and can explain, at least partially, the negative effects generated by the infusion of acid solutions in sickle cell anemia patients.[19] Nevertheless, a simultaneous decrease in pH and deoxygenation, as may occur in the microcirculation, would be negated by the inhibition of the K:Cl efflux and the cells would benefit from an increase in cell volume and a reduction of MCHC, a situation that decreases the tendency of HbS to polymerize. Interindividual variability in the expression of these fluxes (reported by us previously[2,3]) could be a contributor to the interpatient variability in severity observed in this disease.

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Evidence for Direct Action of Human Biosynthetic (Recombinant) GM-CSF on Erythroid Progenitors in Serum-Free Culture

By Anna Rita Migliaccio, Milena Bruno, and Giovanni Migliaccio

The biologic activity of human biosynthetic granulocyte-monocyte colony stimulating factor (GM-CSF) was investigated in serum-free culture of erythroid progenitors derived from adult peripheral blood. The/morphology of erythroid bursts and the cloning efficiency of BFU-E under serum-free conditions were similar to those observed in dishes with fetal bovine serum (FBS). For these experiments, progenitor cells were partially purified by Ficoll-Paque density centrifugation, adherence to a plastic surface, and complement-mediated cytotoxicity of Leu-1$^+$ elements. For some studies, blastlike cells were harvested directly from 6-day-old semisolid cultures. In serum-free culture of the light-density cell fraction, biosynthetic erythropoietin (Ep) was sufficient for formation of pure and mixed erythroid colonies whereas GM-CSF was required for granulocyte-monocytic colonies. When adherent and Leu-1$^+$ cells were removed, or when in vitro differentiated blast cells were used as a source of progenitors, neither Ep or GM-CSF alone induced colony formation. In dishes supplemented with both growth factors, erythroid bursts were detected. Although the presence of GM-CSF alone did not induce formation of any colony or clusters, BFU-E were recorded when Ep was added 8 days later, suggesting that BFU-E could be maintained. Terminal maturation of the resulting erythroid bursts was delayed by 8 days. These results provide evidence that GM-CSF acts directly on early erythroid progenitors. Furthermore, they suggest that both Ep and GM-CSF are necessary to start the differentiation process.

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HUMAN granulocyte-monocyte colony-stimulating factor (GM-CSF) has been identified in the conditioned medium of T lymphocytes responding to lectin and of some tumor cells[1] on the basis of its capacity to induce the formation of colonies composed of granulocytic and monocytic elements in cultures of progenitor cells from human marrow or blood.[2] A 22,000 dalton GM-CSF was purified from medium conditioned by the Mo-T lymphoblastoid line (Mo).[3] Recently, complementary DNA clones have been isolated,[4] and GM-CSF has been obtained following transient cDNA expression in monkey kidney COS cells.[4] The availability of biosynthetic and pure GM-CSF allows in vivo and in vitro analysis of the biologic activity of GM-CSF preparations free from contamination by other growth factors.[5-8]

Previous studies have suggested that biosynthetic GM-CSF preparations have burst-promoting activity (BPA) because they stimulate the formation of erythroid bursts in the presence of erythropoietin (Ep).[5-7] These results have been confirmed with highly purified progenitor cells from adult bone marrow in standard fetal bovine serum (FBS)-supplemented semisolid cultures.[8]

We recently developed serum-free culture conditions that allow optimal growth of human erythroid progenitors in terms of cloning efficiency, colony size and degree of cell differentiation.[9] We used this system to investigate the biologic activity of biosynthetic GM-CSF under circumstances in which serum factors could not play a role.

MATERIALS AND METHODS

Cell preparation. Heparinized peripheral blood from five appropriately informed adult (aged 30 to 35 years) volunteers was diluted 1:1 in Iscove's modified Dulbecco's medium (IMDM) and the light-density cell (LDC) fraction was separated by Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden). Removal of adherent cells was achieved by 2×20-minute adherence to a plastic surface where indicated (mean cellular recovery = 70% to 75%). The final monocyte concentration in nonadherent LDC (LDC-Ad$^-$) was 4% as evaluated by AD acetate esterase staining. Removal of T lymphocytes (LDC-Ad$^-$T$^-$) was accomplished by complement-mediated cytotoxicity after incubation of the LDC-Ad$^-$cells with the anti-human pan-T cytotoxic antibody Leu-1 (Becton Dickinson, Mountain View, CA). In this case, the mean cell recovery was 20% to 30%, and the residual concentration of Leu-1$^+$ cells, evaluated by indirect immunofluorescence, was 5% to 10%.

Hemopoietic growth factors. Either partially purified human Ep (step I, specific activity 1,000 IU/mg protein, Terry Fox Laboratories, Vancouver, Canada) or pure biosynthetic human Ep (specific activity 70,000 IU/mg, Amgen, Thousand Oaks, CA) was used. BPA activity was provided by medium conditioned by the Mo line[3] or by pure biosynthetic GM-CSF obtained from serum-free medium conditioned by COS monkey kidney cells transfected with the GM-CSF cDNA clone in the expression vector p91023 as described.[3,4,6,8]

Control cell culture. Each 1-mL plate contained the following components in IMDM[10]: methylcellulose (0.8%, final concentration), β-mercaptoethanol (7.5×10^{-5} mol/L), a selected heat-inactivated lot of FBS (No. 2919130, Flow laboratories, Glasgow, Scotland) (40%, vol/vol) and nucleated cells (3×10^5 peripheral blood LDC). In some experiments, the LDC-Ad$^-$ or LDC-Ad$^-$T$^-$ cells derived from 3×10^5 peripheral blood LDCs, were plated. An aliquot was counted, and the residual number of adherent and Leu-1$^+$ cells was evaluated (described above). Ep (1.5 to 2 IU/mL) was added where indicated.

Serum-free culture. FBS was substituted by deionized bovine serum albumin (BSA) and BSA-adsorbed cholesterol (final concentrations, 2×10^{-4} mol/L in both cases), iron-saturated transferrin (5×10^{-7} mol/L), insulin (1.7×10^{-6} mol/L), nucleosides (10 μg/mL each), sodium pyruvate (10^{-4} mol/L) and L-glutamine (2×10^{-3} mol/L) as previously described.[9]

Cultures were established in duplicate. Plates were incubated at

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37°C in a fully humidified incubator with 5% CO_2 in air, and erythroid bursts were scored at 12 to 14 days according to standard criteria. The number of erythroblasts in each colony and their degree of maturation (ie, the ratio between immature and mature erythroblasts per dish) was evaluated as described.[9] In some experiments, CFU-GM colonies were also counted. Mean values ± SD from at least three separate experiments are presented.

Colony replating experiments. Clusters of 10 to 20 cells were individually removed in a small culture volume (3 μL) with a thin Pasteur pipette from 6-day-old serum-free cultures of LDC-Ad$^-$ peripheral blood cells stimulated with GM-CSF (10^{-10} mol/L) and Ep (1.5 IU/mL). Each cluster was transferred to a separate microwell containing 200 μL FBS-free culture media supplemented or not with Ep and/or GM-CSF. Some clusters were transferred to Ep-supplemented FBS-rich cultures as a control. The microwells were then scored at 14 days. Colonies containing >500 erythroid cells were considered BFU-E–derived colonies.

RESULTS

Cloning of human BFU-E in serum-free culture. The cloning efficiencies of erythroid progenitors from adult peripheral blood in FBS-rich and serum-free cultures are compared in Table 1. BFU-E cloning was strictly dependent on the presence of Ep. Semipurified urinary (uEp) (data not shown) and biosynthetic (rEp) Ep (Table 1) induced a comparable number of BFU-E–derived colonies in both FBS-rich and serum-free cultures in the absence of an external source of BPA. Addition of Mo-CM or GM-CSF did not increase the number of erythroid bursts (Table 1). This apparent BPA-independent growth of adult erythroid progenitors was not simply due to BPA contamination of some nutrients (ie, BSA), because removal of adherent cells completely abolished this effect (see below).

Effect of GM-CSF and/or Ep on the cloning of human BFU-E from LDC-Ad$^-$ and LDC-Ad$^-$T$^-$ fractions. When the adherent cells were removed, BFU-E cloning under serum-free conditions was dependent on the simultaneous presence of Mo-CM (a source of BPA) and Ep (Fig 1A and B). Some of the BFU-E clones (10% to 20%) were mixed colonies. GM-CSF could also be used as a source of BPA and induced erythroid colony formation in the presence of Ep in a concentration/dependent fashion (Fig 1A). The maximal frequency of erythroid bursts was observed at a GM-CSF concentration of 10^{-10} mol/L and was generally lower than the frequency of bursts detected in cultures supplemented with Mo-CM (Fig 1A). This was not due to a nonspecific effect of the elution buffer used during the GM-CSF purification because its addition to control dishes at a volume corresponding to the maximal GM-CSF concentration used did not affect the BFU-E cloning efficiency (data not shown).

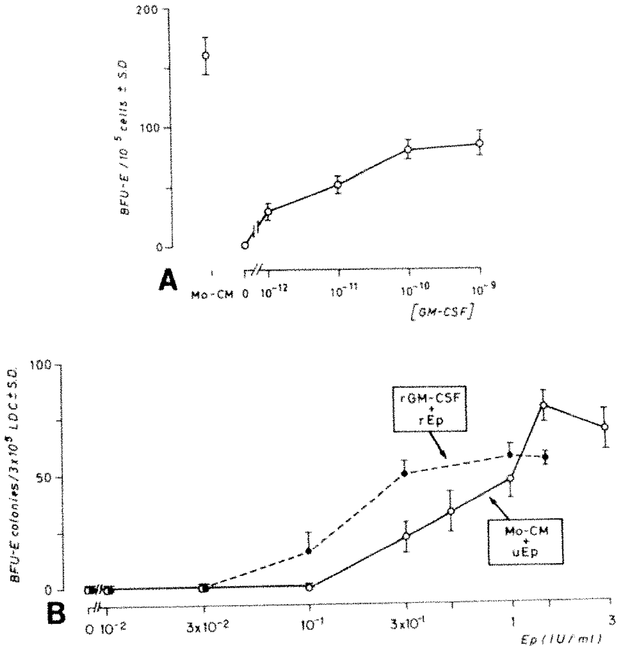

Fig 1. **(A) GM-CSF dose/response of BFU-E–derived colonies from the LDC-Ad$^-$ fraction in the presence of saturating amounts of recombinant Ep (1.5 IU/mL). Mo-CM–supplemented cultures (1%, vol/vol) are presented as controls. The CFU-GM colonies were also scored, and their maximal number per dish was 9.2 ± 4 (GM-CSF, 10^{-9} mol/L + Ep, 1.5 IU/mL), under these serum-free conditions. (B) Natural v biosynthetic Ep dose/response curve on adult BFU-E–derived colonies from LDC-Ad$^-$ fraction of peripheral blood in serum-free cultures supplemented with Mo-CM (10% vol/vol) or GM-CSF (10^{-10} mol/L), respectively. The colony numbers are expressed per 3 × 10^5 LDC.**

The colonies induced by GM-CSF were smaller than the

Table 1. Colonies Generated by Human Progenitors From Normal Adult Blood in FBS-Rich and Serum-Free Culture

		FBS-Rich		Serum-Free	
Cells	Stimulus	BFU-E	CFU-GM	BFU-E	CFU-GM
LDC	—	0	49 ± 7	0	0
(3 × 10^5	rEp	69 ± 3	17 ± 4	70 ± 6	5 ± 5
cells/plate)	Mo-CM	0	46 ± 9	0	35 ± 5
	Mo-CM + rEp	65 ± 5	10 ± 2	67 ± 5	10 ± 1
	GM-CSF	0	42 ± 5	0	19 ± 6
	GM-CSF + rEp	67 ± 3	19 ± 4	63 ± 4	14 ± 3
LDC-Ad$^-$T$^-$	—	0	0	0	0
(6 × 10^4	rEp	17 ± 1	0	0	0
cells/plate)	Mo-CM	0	36 ± 8	0	21 ± 3
	Mo-CM + rEp	48 ± 10	13 ± 7	33 ± 9	1 ± 2
	GM-CSF	0	13 ± 7	0	0
	GM-CSF + rEp	20 ± 5	11 ± 5	25 ± 7	0

Values are mean ± SD from three separate experiments.

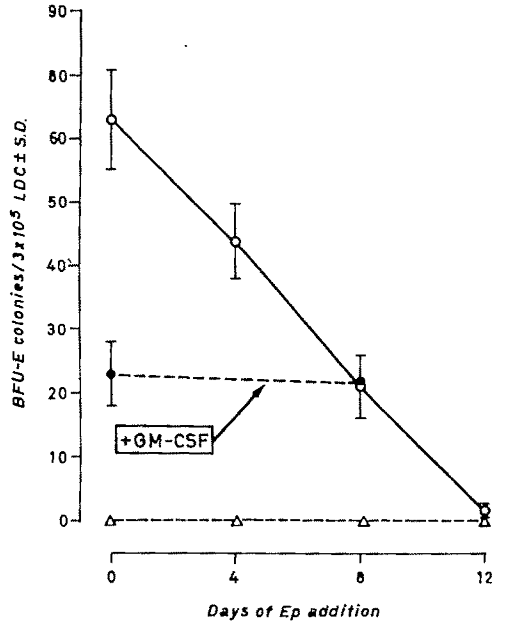

Fig 2. Survival of BFU-E progenitors from the LDC (—O—) or from the LDC-Ad$^-$ (—△—) fraction of peripheral blood upon sequential addition of recombinant Ep (1.5 IU/mL). Ep was added at day 0, 4, 8, or 12 of cultures, and maximal numbers of pink-red erythroid colonies were detected at days 12, 16, 20, and 24, respectively. GM-CSF (10^{-10} mol/L) was added at day 0 in some of the LDC-Ad$^-$ cultures (—●—).

colonies in Mo-CM–stimulated cultures (the mean cellular number per colony was 7 × 10^3 and 10^4, respectively), GM-CSF–induced colonies contained only erythroid cells, and their mean maturation time was 2 days shorter than observed for Mo-CM (the ratio between mature and immature erythroblasts was maximal at 14 to 15 and 16 to 17 days of culture, respectively).

The Ep dose/response curves on progenitor cells derived from the LDC-Ad$^-$ fraction of peripheral blood and stimulated either with Mo-CM or GM-CSF are shown in Fig 1B. uEp was used in Mo-CM–supplemented cultures, and rEp was used in GM-CSF–supplemented cultures. Preliminary experiments did not show significant differences in the dose/response curve of uEp and rEp in FBS–supplemented cultures (data not shown). Similarly, no difference was observed in the recombinant Ep dose/response curve in serum-free culture when adherent cells[9] or GM-CSF (Fig 1B) were used as a source of BPA. The Ep concentration required for 50% of the maximal cloning efficiency in GM-CSF–stimulated LDC-Ad$^-$ cultures, however, appeared slightly lower than the dose required in Mo-CM–stimulated cultures.

The BPA activity of GM-CSF persisted when the Leu-1$^+$ cells were removed (Table 1). Erythroid bursts derived from these LDC-Ad$^-$T$^-$ cells were morphologically equivalent to those obtained from the LDC fraction.

Survival of BFU-E progenitors in serum-free cultures in delayed Ep addition experiments. When Ep was added after 4 or 8 days in culture of the LDC fraction of peripheral blood, more than one third of the original number of BFU-E–derived colonies were observed (Fig 2). Maximal numbers of erythroid colonies were always observed 12 days after the time of Ep addition, however (ie, at 16 or 20 days of culture, respectively) whereas the morphology and the size of the colonies were not affected. The decreased BFU-E cloning efficiency after delayed Ep addition could be due to evolving suboptimal culture conditions with time.

Removal of adherent cells completely abolished the BFU-E survival (Fig 2), suggesting that this effect was mediated by factors produced by macrophages. The addition to the culture at day 0 of GM-CSF (10^{-10} mol/L) partially restored the maintenance of BFU-E in LDC-Ad$^-$ cell cultures (Fig 2).

Formation of erythroid bursts from single clusters under serum-deprived conditions. To clarify whether GM-CSF promoted the growth of erythroid progenitors directly or indirectly by activating contaminating cells to release BPA in vitro, erythroid progenitor cells were purified by collecting clusters of blastlike cells (10 to 20 cells/cluster) from 6-day-old serum-free cultures supplemented with Ep and GM-CSF. No such clusters were seen in dishes supplemented with only GM-CSF or Ep. Each cluster was transferred to a separate microwell and cultured for another 8 days. The results are summarized in Table 2. In FBS-rich cultures, 82% of the transferred clusters gave rise to erythroid colonies. Under serum-deprived conditions, no or very few clusters differentiated without addition of growth factors or in the presence of Ep or GM-CSF alone. The combination of both growth factors gave rise to erythroid colonies with 65% of recloning efficiency.

Effect of GM-CSF on the cloning of human CFU-GM. Under serum-free conditions, the nutrients required to grow murine erythroid progenitors are similar to those required by granulocyte-monocytic progenitors (ref. 11; Magliaccio, Bruno, Magliaccio, unpublished observations). Furthermore, the survival of human erythroid progenitors is dependent on culture conditions more complex than those

Table 2. Erythroid Differentiation From Blast Cell Clusters Generated at Day 6 in GM-CSF–Supplemented Serum-Free Cultures of LDC-Ad$^-$ Peripheral Blood Cells and Singly Transplanted in Microwells

Stimulus in Secondary Cultures	FBS-Rich	FBS-Free			
	Ep	None	rEp	GM-CSF	GM-CSF + rEp
No. of microwells in which cell clusters were singly replated at day 6	11	11	21	23	23
No. of microwells in which >500 erythroid cells were recognized at day 14	9	0	3	1	15
Percentage of recloning	82	0	14	4	65

A representative experiment is presented.

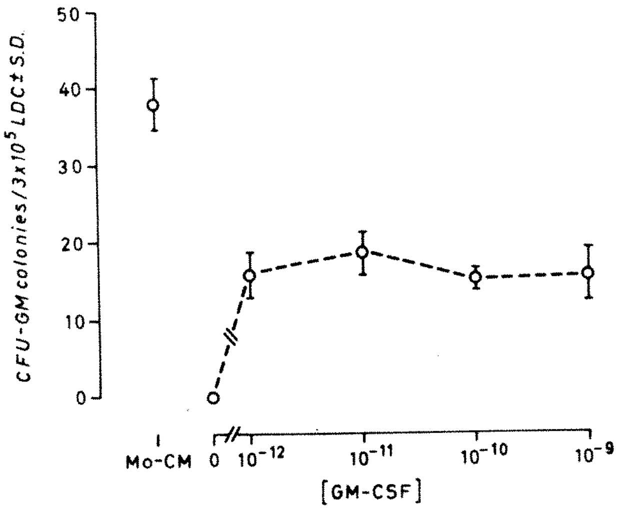

Fig 3. GM-CSF dose/response curve on CFU-GM-derived colonies from the LDC fraction of peripheral blood.

required by human CFU-GM.[12] For these reasons, we used the serum-free conditions that we developed for human BFU-E to culture human CFU-GM.

When the LDC fraction of peripheral blood was used as source of progenitor cells, the cloning of CFU-GM under serum-free conditions was strictly dependent on addition of GM-CSF (Fig 3). Maximal number of granulocyte-monocytic colonies were observed at a GM-CSF concentration of 10^{-12} mol/L (Fig 3), which has very low BPA (Fig 1A). Both monocyte and/or granulocyte colonies (5×10^2 cells/colony), morphologically identical to those obtained in cultures supplemented with FBS were detected.

When adherent and Leu-1^+ cells were removed, GM-CSF was sufficient to induce CFU-GM colony formation in FBS-rich but not in serum-free cultures (legend to Fig 1A and Table 1).

DISCUSSION

Biosynthetic GM-CSF has multilineage stimulatory activities because it supports the formation of colonies along the macrophage, granulocyte, and erythroid differentiation pathways in FBS-supplemented culture.[8] Some of the erythroid colonies also contain granulocytic and macrophagic elements and this led to the conclusion that human GM-CSF has an effect on the proliferation and/or differentiation of multipotential progenitor cells.[8]

Formation of hematopoietic colonies in culture depends on the interplay of several factors. These factors may derive from accessory cells or be present in FBS. For these reasons, we investigated the BPA of biosynthetic GM-CSF in serum-free cultures of progenitor cells obtained from adult peripheral blood.

Under serum-free conditions, the LDC fraction of adult peripheral blood (as well as the unfractionated bone marrow, unpublished observations) produced sufficient BPA endogenously to sustain maximal BFU-E cloning in the presence of Ep (Table 1). These systems were therefore not suitable to investigate BPA. When adherent and Leu-1^+ cells were removed, however, no BFU-E-derived colonies were observed in serum-free cultures of peripheral blood (Fig 1A and Table 1), although such colonies persisted in cultures of marrow cells (data not shown; also ref. 13). For this reason, blood was chosen as source of progenitor cells to investigate the BPA of recombinant GM-CSF.

In serum-free cultures of Ad^-T^- blood cells, GM-CSF alone did not induce the formation of colonies or clusters, although Ep and GM-CSF together induced the formation of *pure* erythroid bursts. The number of erythroid bursts was dependent on the concentrations of both Ep and GM-CSF. The lack of CFU-GM-derived colonies in GM-CSF-supplemented serum-free cultures of partially purified progenitor cells is difficult to interpret. It may reflect either culture conditions nonoptimal in terms of nutrients or growth factor(s) or the presence of inhibitory factor(s) released from residual accessory cells. As shown in Table 1 and Fig 1A, the maximal number of erythroid bursts induced by GM-CSF in the presence of Ep was less than the number observed in serum-free cultures supplemented with Mo-CM and Ep. Similarly, under FBS-rich conditions, fewer bursts have been reported in cultures of human[8] or murine[14] cells stimulated with Ep and GM-CSF than in cultures stimulated with Ep and conditioned media. Because GM-CSF-induced colonies were smaller and matured 2 days earlier than colonies induced by Mo-CM, they may represent only a fraction of the total BFU-E population in human peripheral blood, perhaps the most differentiated one.

Progenitor cells represented 0.1% of the LDC-Ad^-T^- cell fraction and 5% of the monocytes, and 5% to 10% of the T lymphocytes were not removed by the purification procedure. The effects on BFU-E observed in the presence of GM-CSF may have been indirectly mediated through stimulation of BPA production by accessory cells. GM-CSF sustained the differentiation of erythroid colonies from single clusters of blast cells in the presence of Ep, however, suggesting a direct effect.

No colony or cluster was observed in cultures of LDC-Ad^- cells in the presence of GM-CSF. Experiments with delayed Ep addition indicate, however, that the erythroid progenitors were maintained for at least 8 days in these dishes (Fig 3). When Ep was added in these GM-CSF-supplemented cultures, erythroid colonies were detectable 12 days later. This last result suggests that the process of erythroid differentiation in its early phases requires the presence of both factors.

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Human Blood Basophils Display a Unique Phenotype Including Activation Linked Membrane Structures

By Christoph Stain, Hannes Stockinger, Michael Scharf, Ulrich Jäger, Heinz Gössinger,
Klaus Lechner, and Peter Bettelheim

To evaluate the membrane marker profile of human basophils a panel of well-established monoclonal antibodies (MoAbs, $n = 60$) was used for a combined toluidine/immunofluorescence staining procedure. Myeloid-associated MoAbs (particularly MoAbs against the LFA-1 family (CD11, CD$_w$18), MoAbs directed against lactosylceramide (CD$_w$17), anti-glycoprotein (gp) 150 MoAbs MCS 2 and MY 7 (CD$_w$13), anti-gp 67 MoAb MY 9, anti Fcγ-receptor (mol wt 40 kd) MoAb CIKM5, anti-CR 1 MoAb E 11, and the antiglycolipid MoAb VIM-2) were reactive with basophils, indicating a close relationship to other mature myeloid cells. Under normal conditions, basophils surprisingly express at least three activation-linked structures not detectable on mature neutrophils, ie, the p45 structure defined by MoAbs OKT-10 and VIP-2b, the p24 structure identified by the CD9 MoAb BA-2, and the receptor for interleukin 2 (IL 2) recognized by three different MoAbs (anti-TAC, IL2RI, anti-IL 2). Moreover, under short-term culture conditions basophils both in mononuclear cell (MNC) suspension and as purified fractions display the HLA-DR and T4 antigens. The neutrophilic/eosinophilic structure 3-fucosyl-*N*-acetyllactosamine is expressed on basophils only after neuraminidase treatment. Basophils were not stained at all by CD16 MoAbs directed against the Fc γ-receptor (mol wt 50 to 70 kd) of neutrophils, by the MoAb 63D3 (CD$_w$12) recognizing the monocyte/granulocyte-associated p 200 antigen, and by the CD$_w$14 antibodies (VIM-13, Mo 2) defining the monocyte-specific structure p 55. Enriched basophils freshly obtained from chronic granulocytic leukemia (CGL) patients yielded identical results in FACS analyses. In summary, these data indicate that basophils generate a unique combination of surface determinants and possibly represent an activated cell population.

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HUMAN BASOPHILS represent a distinct minority of peripheral blood cells with a prevalence of 0.5% of total leukocytes.[1] Mature basophilic cells have to be distinctly differentiated from neutrophils and eosinophils in terms of their physiologic role. So far, evidence of basophil differentiation was mostly restricted to the identification of morphological characteristics and biochemical activities.[2-7] In contrast to neutrophils and eosinophils, the expression of membrane antigens has hitherto not been investigated in detail.[8-11] This is due to the small amount of basophils in the peripheral blood and to the fact that basophils, which unlike neutrophils and eosinophils remain in mononuclear cell (MNC) preparations, cannot be distinguished from other cells in MNC analyses unless additional cytochemical staining procedures are performed. Using a combined cytochemical (toluidine) and immunologic (monoclonal antibodies, MoAbs) staining procedure, we were able to analyze the antigenic membrane profile of this cell type.

MATERIALS AND METHODS

Cell Preparation

MNC were isolated by gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and were then subjected to staining with MoAbs. Neutrophils obtained from healthy controls ($n = 3$) were prepared by standard techniques as previously described.[12] Eosinophils were isolated from two patients with hypereosinophilia and from two patients suffering from transient drug allergy. Purity of these cell fractions was >90%.

MoAbs

The MoAbs used in this study and referred to as belonging to a cluster of differentiation (CD) or for being reactive with a well-defined structure were: Leu 1 (CD5), Leu 2a (CD8), Leu 3a (CD4), Leu 4 (CD3), Leu 7 [human natural killer (NK) cells], Leu 11a (CD16), and anti-HLA-DR (Becton Dickinson, Sunnyvale, CA); MY 7 (CD$_w$13), MY 9 (anti-p67), Mo 2 (CD$_w$14), T4 (CD4), T8 (CD8), T11 (CD2), B1 (CD20), B2 (CD21), B4 (CD19), and IL 2 R1 (CD25) (Coulter Immunology, Hialeah, FL); BA-1 (CD24), and BA-2 (CD9) (Hybritech, San Diego); OKT-1 (CD5), OKT-9 (antitransferrin receptor), OKT-10 (p45), OKM-1 (CD11), and OKB-2 (CD24) (Ortho Pharmaceuticals, Raritan, NJ); and 63D3 (CD$_w$12) (BRL; Gaithersburg, MD); anti-Il 2 (Eurogenetics, Agoralaan, Belgium); BMA-022 (HLA-DR) and BMA-0111 (PanT-ERFC receptor) (Behringwerke AG, Marburg, FRG).

The MoAbs VIP-1 (antitransferrin receptor),[13] VIP-2b (p45),[13] VID-1 (anti-HLA-DR),[14] VIL-A1 (CD10),[15] VIM-12 (CD11),[16] VIM-13 (CD$_w$14),[16] VIM-D5 (CD15),[12] VIM-2 (not clustered),[17] VIM-C6 (CD15),[18] VIB-C5 (CD24),[19] VIT-3b (CD3),[20] VIT-4 (CD4),[19] VIT-6 (CD1),[21] VIT-8 (CD8),[19] VIT-12 (CD6),[19] ViFcR2 (CD16), and VIE-G4 (anti-glycophorin A)[22] were produced at the Institute of Immunology, University of Vienna. The MoAb Na1/34 (CD1) was kindly provided by Dr A. J. McMichael. WT-1 (CD7) antibody was sent by Dr W. I. M. Tax, and MCS-2 (CD$_w$13) antibody was donated by Dr J. Minowada. VEP-13 (CD16) was supplied by Dr Rumpold (Vienna). Anti-TAC (CD25) was kindly

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provided by Dr T. A. Waldmann. CLBery3 was sent by P. T. Tetteroo. MoAbs L4F3, 1G10 (CD15), KiM5 (CD11), T5A7 and (G)035 (CD17), TSI/18.11 and MHM 23 (CD$_w$18, LFA-1-β-chain; McMichael, personal communication, 1984, 1986), CIKM5 (40 kd Fcγ-receptor) were selected from the myeloid panel of the Second International Workshop on Human Leukocyte Differentiation Antigens. MoAb E 11 (anti-CR 1 receptor) was taken from the myeloid panel of the Third International Workshop on Human Leukocyte Differentiation Antigens.

Supernatant from the IgE-producing human myeloma cell line U-266 and a mouse anti-human IgE MoAb (E124 2-8, Immunotech, Marseille, France) was used for detection of membrane-bound IgE (positive control). Monoclonal antibodies of either IgG 1, IgG 2a, IgG 2b, and IgM subclass with irrelevant specificity were used as negative controls.

Immunologic Characterization With MoAbs

As previously described,[12] the binding of the various antibodies to isolated MNCs was assessed by indirect immunofluorescence with fluoresceinated goat F(ab')$_2$ anti-mouse IgG and IgM antibodies. Fluorescence of MNCs was evaluated by means of a Leitz Ortholux microscope (Leitz, Wetzlar, FRG). Purified basophils of CGL patients and enriched neutrophils of one healthy donor were analyzed with a FACS 440 (Becton Dickinson).

Combined Immunologic/Toluidine Staining Procedure

Blood samples from 15 volunteers were drawn for testing the entire panel three times. After the indirect immunofluorescence staining procedure, cells (6×10^5 for each MoAb) were fixed in glutaraldehyde at room temperature (RT) for 1 minute (0.025% glutaraldehyde in fixation buffer: 0.1 mol/L Tris buffer in 1 vol% glucose, pH 7.8). After three washings with phosphate-buffered saline (PBS, pH 7.2) cells were incubated with toluidine blue (Sigma, St Louis) (0.0125% wt/vol in PBS) at RT for 15 minutes. Subsequently, cells were washed three times in PBS and subjected to analysis as follows: Toluidine-stained cells were first identified in bright field and then examined for antibody binding under fluorescence light.

The antibodies we used either stained practically all cells examined or did not stain basophils at all. For FACS analyses, a reactivity of >20% was considered positive.

Neuraminidase Treatment of Cells

MNCs (4×10^6/mL) were incubated with *Vibrio cholerae* neuraminidase 0.2 U/mL (Behringwerke AG) in a shaking water bath at 37°C for 30 minutes. The reaction was terminated by washing the mixture three times with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Enrichment of Basophils

To compare the phenotype of normal basophils with that of CGL patients (two patients in stable phase, one patient in accelerated phase), the combined immunofluorescence technique and an analysis of enriched basophils was used. Enrichment of basophils was achieved by incubating MNC with a mixture of antibodies and subsequent rabbit complement lysis. After informed consent was obtained from each patient, heparinized peripheral blood of three CGL patients (5%, 11%, and 15% basophils, respectively) underwent MNC isolation as described above; 5×10^8 cells were incubated with 1 mL RPMI at 4°C for 45 minutes, the milliliter of RPMI containing 100 μg of MoAB VIM-D5. After being washed, cells were exposed to 5 mL rabbit complement (Behringwerke AG) at 37°C for 90 minutes. Monocyte depletion of washed cells was achieved with scrubbed nylon wool (37°C for 45'). Cells were then incubated with a mixture of antibodies consisting of 25 μg VIT-3, 25 μg VIB-C5, 25 μg Leu 1, 50 μg Leu 7, 25 μg VIE-G4, 25 μg anti-HLA-DR, CLBery3 (ascites, 1:500 diluted), 25 μg VIM-D5 at 4°C for 45 minutes, and subsequently incubated with 4 mL rabbit complement at 37°C for 90 minutes. Cells were again centrifuged on Ficoll-Hypaque and washed twice. Cytospin preparation of this cell fraction revealed >95% basophils (Fig 1).

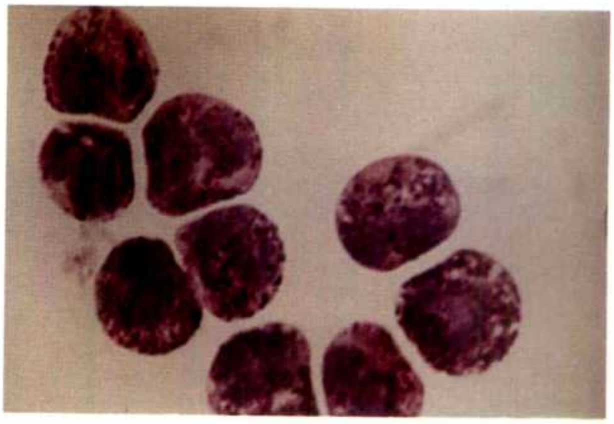

Fig 1. Purified CGL basophils obtained by MoAb + complement lysis.

Culture Studies

Activation of neutrophils and basophils by formyl-methionyl-leucyl-phenylalanine (fMLP). As reported previously,[23] PMNs (1×10^7/mL) or MNCs (1×10^7/mL) were incubated at 37°C for 10 minutes, the medium (RPMI 1640, 5 g/L human albumin, pH 7.2) containing 1 μmol/L fMLP and cytochalasin B 10 μg/mL as well as dimethylsulfoxide (DMSO) (final concentration 0.01%) as a solvent. The reactivity of stimulated MNCs was evaluated by means of the combined immunofluorescence/toluidine staining procedure.

Activation of basophils from two healthy donors (in MNC suspension) with the Ca-ionophore A23187. Cells (1×10^7) were incubated with the Ca-ionophore A23187 (1 μg/mL, Sigma) at 37°C for 30 minutes. After this procedure degranulation of basophils was evident.

Short-term cultures with biologic factors. Basophils were cultured either in MNC suspension (five healthy controls) or as purified fractions (CGL basophils from two patients) after lysis with a mixture of antibodies (>95% purity as determined by Wright staining of cytospin preparations) for 5 days in RPMI 1640 (Flow Laboratories, McLean, VA) plus 10% FCS, supplemented with either 20 U/mL human recombinant IL 2 (Boehringer Mannheim), 100 U/mL human recombinant γ-interferon (hr-γ-IFN, Interferon Sciences, New Brunswick, NJ), supernatant of the HTB-9 cell line[24] (9:1 vol/vol), or supernatant of the T cell hybridoma producing basophil-like promoting activity factor (BaPA, 4:1 vol/vol) kindly provided by Dr B. Stadler.[25,26] Phenotyping (normal basophils in MNC fractions: combined toluidine immunofluorescence technique; purified CGL basophils: FACS analyses) of cultured cells was performed on days 0, 2, and 5.

^{3}H-incorporation. Incorporation of ^{3}H-thymidine was carried out as described previously.[27] In brief, purified basophils from two CGL patients (10^5 cells/well) were incubated with factor-conditioned media (γ-IFN, BaPA, HTB-9-supernatant, and IL 2) at 37°C for up to 5 days in a humidified atmosphere with 5% CO$_2$. One

microcurie per well ^{3}H-thymidine (New England Nuclear) was added 12 hours before harvest. The incorporated radioactivity was collected on glass-fiber filters and counted in a liquid scintillation counter. Values are given as cpm $\times 10^{-3} \pm$ SD of triplicate cultures.

Enrichment of neutrophils by MoAb + complement lysis. Heparinized blood drawn from a normal donor was first centrifuged on Ficoll-Hypaque. The sedimented cell pellet containing erythrocytes, neutrophils, and residual MNCs, subsequently underwent dextran sedimentation. Neutrophils (1×10^8) obtained by this method were incubated with a mixture of MoAbs comprising 25 μg anti-HLA-DR, 25 μg BMAO111, 25 μg B1, 25 μg VIM-13, and CLBery3 (ascites, 1:500 diluted) at 4°C for 45 minutes. Cells were then exposed to 1 mL rabbit complement at 37°C for 90 minutes. After two washings, cytospin examination revealed >95% neutrophils.

RESULTS

By means of a toluidine staining procedure in suspension, basophils are easily identified by their purple cytoplasmic granules. This procedure, combined with the simultaneous application of the immunofluorescence technique allowed determination of the basophil marker profile (Fig 2A and B).

A panel of 60 MoAbs was used to delineate the phenotype of basophils from that of neutrophils and eosinophils. Thirty MoAbs reacted with at least one type of mature granulocytes and showed a homogeneous staining for positive subsets in all samples tested. Table 1 shows that 20 MoAbs stained basophils. MoAbs giving positive results and identifying common granulocytic membrane structures were, in particular, anti-CR1 MoAbs (220 kd), anti-Fcγ-receptor (40 kd) MoAbs, anti-C3bi-receptor antibodies (OKM-1, VIM-12, and Ki M5/CD 11), anti-LFA-1-β chain MoAbs (CD_w18), anti-gp 150 MoAbs (CD_w13), and MoAb VIM-2, which recognizes a glycolipid structure. Of all MoAbs tested in this study, antilactosylceramide MoAbs (CD17) showed the strongest reactivity with basophils (Fig 2A and B). MoAbs detecting the p 67 membrane determinant bound to basophils moderately and, in our hands, gave a very weak reactivity with neutrophils as well.

In contrast to eosinophils and/or neutrophils, the structure 3-fucosyl-*N*-acetyllactosamine (identified by MoAbs clustered as CD15) and the 50- to 70-kd Fcγ-receptor (recognized by CD16 MoAbs) were not detectable on the surface membrane of circulating basophils. The CD_w12 and CD_w14 anti–monocyte-associated MoAbs tested also failed to react with basophils.

We furthermore tested nonmyeloid-associated MoAbs of clusters CD1-10, 19 through 21, 24, 25, MoAbs directed against the T10 antigen and an antitransferrin receptor MoAb. A constant and reproducible finding was the reactivity of various activation-linked markers with basophils. All three anti-IL 2 receptor MoAbs (CD25) tested, both anti-T10 MoAbs, and the anti-p24 MoAb BA-2 (CD9) gave positive results. HLA-DR antigens and the transferrin receptor, also activation-linked structures, were not detectable on freshly obtained basophils. MoAbs directed against lymphocyte-associated structures (CD 1 through 8, 10, 19 through 21, 24, anti-human NK cell MoAb Leu7) failed to stain basophils at all. They included anti-common acute lymphocytic leukemia (cALLA) MoAb VIL-A1 (CD10) and CD24 MoAbs known to bind to neutrophils.

Neuraminidase Treatment of MNCs

Seven MoAbs (VIM-D5, BA-2, T5A7, VEP-13, VID-1, VIP-1, and anti-TAC) were tested after neuraminidase

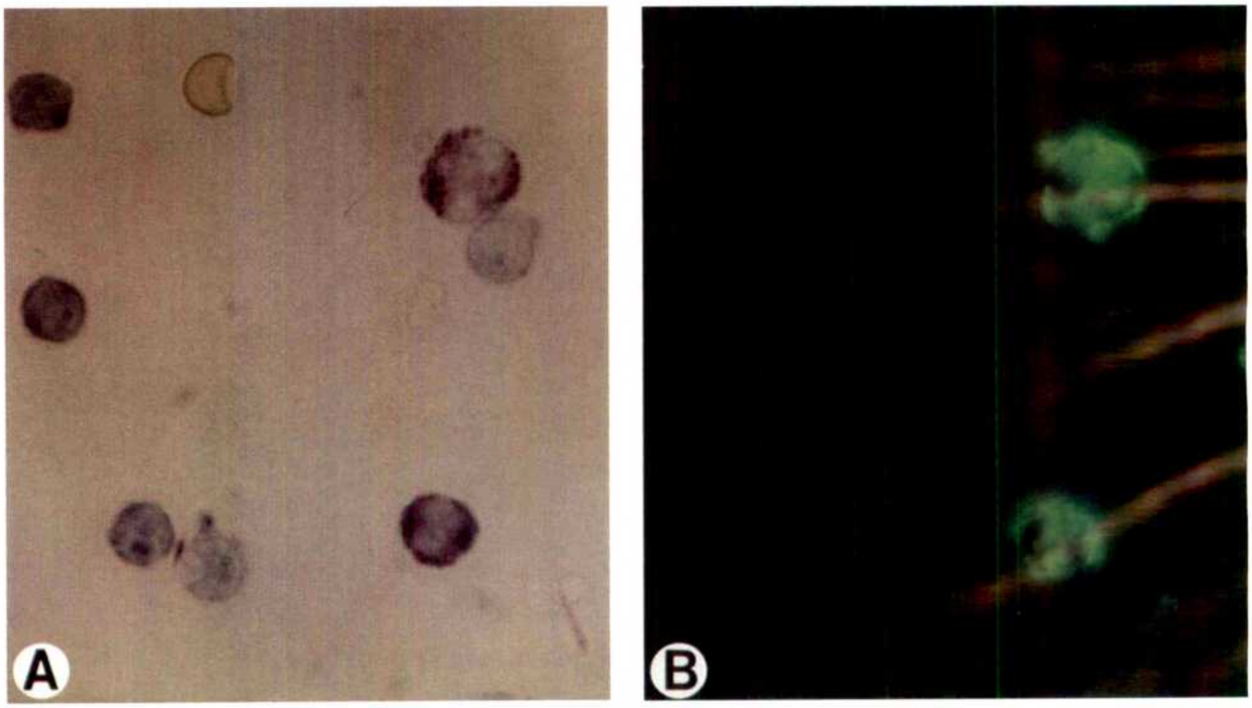

Fig 2. (A and B) Reactivity of the MoAb T5A7 with toluidine-stained peripheral blood basophils.

Table 1. Reactivity Pattern of MoAbs Binding to Mature Granulocytes

MoAbs Reactive With Peripheral Blood Granulocytes*	WHO-Cluster/ Reactive Structure	Basophils	Neutrophils	Eosinophils
OKM-1 VIM-12 KIM 5	CD11/CR3	+	++	+
MCS2 MY 7	CD$_w$13/p150	+	+	+
TSI/18.11 MHM 23	CD$_w$18/LFA1β-chain	+	+	+
E11	p220/CR1	+	+	+
CIKM5	40-kd Fc γ-receptor	+	+	+
VIM-2	NK	+	+++	+++
T5A7† (G)035†	CD17/lactosylcer	++	+	–
MY 9 L4F3	p67	+	±	–
VIM-D5 VIM-C6 1G10	CD15/3-FAL	–	+++	++
VIB-C5 BA-1 OKB-2	CD24	–	+	+
VIL-A1	CD10/cALLA	–	+	–
63D3	CD$_w$12	–	+	–
VEP-13 Leu 11a ViFcR2	CD16/50- to 70-kd-Fcγ-receptor	–	++	–
OKT-10 VIP-2b	p45/T10	+	–	–
α-TAC IL 2R1 α-IL 2	CD25/IL 2 receptor	+	–	–
BA-2	CD9/p24	++	–	++

NK, not known.

*Each antigranulocytic MoAb showed a homogeneous staining for positive subsets in all samples tested.

†Reactive with eosinophils in hypereosinophilic syndrome.

treatment of cells. When pretreated with neuraminidase basophils, which are normally VIM-D5 (anti–3-FAL) negative, reacted strongly with this very MoAb. All other MoAbs tested displayed the same reactivity pattern with basophils both before and after neuraminidase treatment.

Purification and Phenotypic Analysis of CGL Basophils

After MoAb plus complement lysis, purity of basophils was >95% (Fig 1). Purified cells underwent indirect immunofluorescence staining with MoAbs of major significance and were evaluated by FACS analyses (Fig 3A and B). Identical results were obtained with all three CGL patients on day 0. These FACS data therefore confirmed the results obtained with normal basophils by use of the combined toluidine staining procedure.

Phenotypic Analysis of Purified Neutrophils

The phenotype of neutrophils has already been well defined. To examine whether the MoAb + complement lysis may cause an alteration of surface structures, purified neutrophils were stained with a panel of MoAbs and measured on a FACS. The phenotype of neutrophils appeared to remain unchanged by this procedure and was identical to the neutrophil reactivity pattern obtained by indirect immunofluorescence microscopy (data not given).

Culture Studies

fMLP effect on basophils and neutrophils. The binding of MoAbs (VIM-D5/CD15, T5A7/CD$_w$17, BA-2/CD9, VID-1/HLA-DR, VIP-1/transferrin receptor, and anti-TAC/IL 2 receptor) was tested before and after fMLP activation of basophils and neutrophils (two healthy controls). Neutrophils displayed a distinct increase of the fluorescence intensity with MoAb VIM-D5, whereas T5A7 bound neutrophils to a lower extent after this procedure. With regard to basophils, no change of fluorescence intensity was evident with any MoAb tested.

Effect of Ca-ionophore A 23187 on basophils. When basophils, in MNC preparations, were exposed to the Ca-ionophore A23187, the reactivity of the MoAbs studied remained unchanged (same MoAbs were tested as are listed in the fMLP experiment).

CGL basophils cultured in factor-conditioned media. Purity of 5-day-cultured basophils still was >95% in all samples tested (as determined by morphological criteria). Trypan blue staining revealed a viability of at least 98% of cells. FACS analyses of basophils obtained from two CGL patients were performed on days 0, 2, and 5. The reactivity of anti-TAC, already detectable on unstimulated cells, was increased on days 2 and 5 and was not dependent on the addition of biologic factors. An example of this reactivity pattern is given in Fig 3C. The HLA-DR and T4 antigens, expressed neither on basophils obtained from healthy donors nor on CGL basophils on day 0, became detectable on days 2 and 5. All three surface structures in question were also identified on cells in the control medium. Table 2 shows a comparison of the anti-TAC, anti–HLA-DR, and T4 reactivities of basophils cultured in the control medium (as determined by FACS analyses on days 0, 2, and 5). The expression of HLA-DR was stronger on BaPA and γ-IFN–stimulated basophils than on those in the other factor-conditioned media and the control medium, respectively (Fig 3D). Moreover, a moderate reactivity of the three anti-T4 antibodies (VIT-4, T4, Leu 3a) was noted in all culture samples, which is shown in Fig 3E. The reactivity of all other MoAbs tested remained unchanged (data not shown).

Normal basophils cultured in factor-conditioned media. Phenotypic analyses (combined toluidine/immunologic staining procedure) of normal basophils from all but one donor (in MNC preparations) cultured for 5 days in

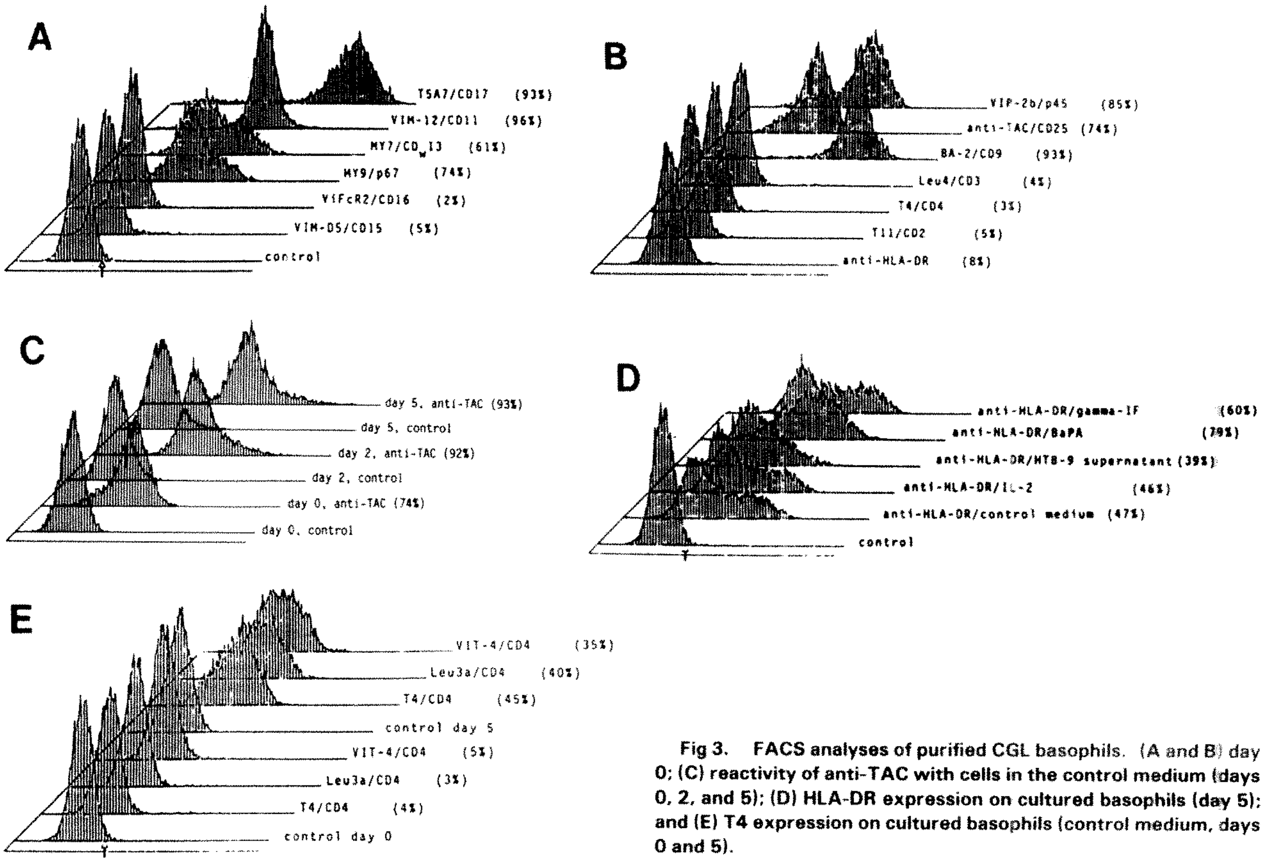

Fig 3. FACS analyses of purified CGL basophils. (A and B) day 0; (C) reactivity of anti-TAC with cells in the control medium (days 0, 2, and 5); (D) HLA-DR expression on cultured basophils (day 5); and (E) T4 expression on cultured basophils (control medium, days 0 and 5).

factor-conditioned media gave identical results (HLA-DR$^+$, T4$^+$) as compared with purified CGL-basophils.

^{3}H-Incorporation studies. Table 3 shows the proliferation kinetics as measured by ^{3}H-thymidine uptake on days 0, 2, and 5. It is evident that the ^{3}H-thymidine incorporation was higher on day 2 than on day 5. A distinct increase of the ^{3}H-thymidine incorporation was noted in the HTB-9–conditioned medium. As compared with the control medium, all other samples revealed no significantly elevated ^{3}H-uptake over a period of 5 days.

DISCUSSION

In this study, toluidine-stained cells were phenotyped with a panel of MoAbs to evaluate the antigenic membrane profile of peripheral blood basophils. The results clearly indicate that basophils express a unique pattern of membrane determinants.

The most conspicuous finding was the detection of three activation-linked structures on the surface membrane of basophils. The presence of the structures in question, ie, the IL 2 receptor, the T10 antigen, and the structure p 24, is consistent with the common idea of basophils representing an activated cell type and/or a population responding immediately to certain stimuli.

So far, reactivity of MoAbs directed against the IL 2 receptor was observed with activated T and B lymphocytes as well as monocytes stimulated by γ-IFN.[28-30] With the exception of the recently established eosinophilic cell line EoL[31] and a subset of myeloid blast cells,[32,33] no granulocytic cells

Table 2. Expression of Surface Determinants on CGL Basophils Cultured in Control Medium as Measured by FACS Analyses

Patient No.	Antibody Designation	Days 0 Reactive (%)	 2 Reactive (%)	 5 Reactive (%)
1	Anti-TAC	74	92	93
	Anti-HLA-DR	8	21	47
	T4	3	18	45
	Control	0	0	0
2	Anti-TAC	59	67	77
	Anti-HLA-DR	4	29	41
	T4	2	23	43
	Control	0	0	0

Table 3. ^{3}H-Thymidine Incorporation of CGL Basophils on Days 0, 2, and 5

Medium	Patient No.	Days 0	 2	 5
Control medium	1	0.6 ± 0.05	5.2 ± 0.3	2.4 ± 0.2
	2	0.5 ± 0.2	4.8 ± 0.9	0.7 ± 0.06
HTB-9	1	0.9 ± 0.05	13.6 ± 0.8	4.8 ± 0.3
	2	0.7 ± 0.07	8.2 ± 0.5	3.3 ± 0.2
γ-IF	1	NT	NT	NT
	2	0.6 ± 0.05	4.3 ± 0.8	0.8 ± 0.02
IL-2	1	0.4 ± 0.02	4.7 ± 0.2	1.9 ± 0.3
	2	0.5 ± 0.06	5.4 ± 0.2	0.7 ± 0.02
BaPA	1	0.6 ± 0.03	8.2 ± 0.8	2.4 ± 0.1
	2	0.6 ± 0.1	3.7 ± 0.3	0.8 ± 0.1

NT, not tested. Values are given in cpm × 10^{-3} ± SD.

have yet been reported to be reactive with anti-IL 2 receptor antibodies.

Basophils also reacted with MoAb BA-2 (CD9), recognizing the p 24 structure. This antibody, which in our hands fails to react with neutrophils, was initially reported to identify a lymphohematopoietic cell surface structure.[34] Recently, the structure p 24 has also been detected on activated T cells,[35] and from affinity-purified antigen preparations it is now evident that the p 24 molecule is responsible for a protein kinase and for growth-promoting properties.[36]

Moreover, basophils were stained by MoAbs detecting the T10 antigen, which represents a new and quite unexpected observation. Whereas this structure was reported to be expressed on immature precursor cells as well as on activated lymphocytes, maturing myeloid cells have so far been considered unreactive with anti-T10 antibodies.[37,38]

Based on the assumption that basophils represent cells in an activated state or that these cells rapidly respond to stimuli, we also studied the basophil phenotype after in vitro culture. To this end, normal basophils in MNC suspension and CGL basophils as purified fractions were exposed to fMLP/cytochalasin B and Ca-ionophore and cultured for 5 days in medium containing either hrIL 2, hr-γ-IFN, supernatant of the HTB-9 cell line (which is reported to enhance blast colony formation),[24] or BaPA synthesized by a T cell hybridoma.[25,26] As compared with freshly obtained normal basophils and CGL basophils, the phenotype of cells in short-term cultures differed in three respects: First, after 2 days of culture, the reactivity of anti-TAC was significantly increased in all samples tested and remained unchanged until day 5. Second, the expression of HLA-DR was detectable on days 2 and 5. Third, the T4 antigen became demonstrable with three different MoAbs directed against this structure. At this point, we cannot definitely say whether the expression of HLA-DR and T4 on basophils is a phenomenon restricted to culture conditions or whether this may also occur in vivo. A differentiation to a "late" HLA-DR–positive state may be conceivable with regard to the other activation-linked structures. Whether the expression of differentiation structures or the susceptibility of receptors corresponds with mediator release requires further investigation.

The expression of various activation-linked structures and the T4 antigen on basophils again raises the question of a possible relationship of T lymphocytes and metachromatically stained cells.[39] The detection of the surface structures in question suggests that the functional properties and/or the differentiation of basophils are influenced by T cells or T cell modulating factors. Despite the occurrence of the T4 antigen on cultured basophils, the existence of a common precursor cell, as proposed by Burnet,[40] seems unlikely with regard to the numerous myeloid-associated determinants. The detection of the T4 antigen on cultured basophils does not represent a novel finding concerning myelomonocytic cells, nor does the presence of this antigen provide evidence for a common precursor since monocytes and γ-IFN–stimulated HL-60 cells were reported to display the T4 antigen as well.[41,42]

The myeloid determinants we detected on basophils were in particular the CR3 receptor recognized by CD11 MoAbs, the structure p150 (CD_w13 MoAbs), the β chain of LFA-1 (CD_w18 MoAbs), lactosylceramide (CD17 MoAbs), the CR1 receptor (MoAb E11), the 40-kd Fcγ-receptor (MoAb CIKM5), and the glycolipid structure identified by MoAb VIM-2, present on all granulocytic cells beyond the stage of promyelocytes. Moreover, basophils clearly displayed the structure p67 (MoAbs MY9, L4F3), which had so far been known to be generated only by myeloid progenitors and monocytic cells.[43]

The p67 determinant was reported to be lost gradually with ongoing granulocyte differentiation.[43] Because staining of basophils was evident in our study, the decrease of antibody binding seems to be restricted to neutrophil and eosinophil differentiation. Basophils we obtained from healthy adults therefore showed a different reactivity with MY9, as did in vitro-cultured fetal basophilic cells which were reported to be MY9 negative.[44]

Certain well-defined neutrophil-associated structures were missing: The structure 3-fucosyl-*N*-acetyllactosamine identified by CD15 MoAbs (VIM-D5, 1G10, VIM-C6) was not detectable. After neuraminidase treatment, however, 3-FAL is expressed on basophils, which represents a phenomenon we already observed with myeloid and lymphatic blast cells.[18] An observation of apparent functional importance was that basophils lack the 50- to 70-kd Fcγ-receptor (recognized by MoAbs VEP-13, Leulla, ViFcR2). This receptor is of major importance for the phagocytotic capacity of neutrophils. Antibodies against this structure completely block the binding and ingestion of IgG-coated sheep erythrocytes.[45] Because basophils are known to bind aggregated human IgG,[46] minor differences of the receptor's molecular structure on the different types of granulocytes could be an explanation for the nonreactivity of the CD16 MoAbs with basophils. An alternative possibility which cannot yet be excluded either is that the 40-kd Fcγ-receptor (recognized by MoAb CIKM5) is the sole IgG binding site of basophils.

CR 1 and the LFA-1 structures, which are further receptors contributing to the functional process of phagocytosis mainly by providing attachment to target particles, were identified by corresponding MoAbs on all types of mature granulocytes. These data confirm previous findings of the CR 1 receptor on basophils, obtained by use of complement-coated particles,[47] and are consistent with the role assigned to basophils in the immune response.

So far, basophils have been explored mostly by morphological,[2,3,48,49] histochemical,[4,7,48] and functional[5,6,50,51] analyses. These studies were laborious and, with a few exceptions, could not be performed simultaneously. The knowledge of the basophil phenotype and the technique used in this study offers a new possibility for the simultaneous determination of differentiation and function and provides the opportunity to influence this cell type specifically through its different surface structures.

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Experimental Transmission and Pathogenesis of Immunodeficiency Syndrome in Cats

By Edward A. Hoover, James I. Mullins, Sandra L. Quackenbush, and Peter W. Gasper

We describe the identification, experimental transmission, and pathogenesis of a naturally occurring powerfully immunosuppressive isolate of feline leukemia virus (designated here as FeLV-FAIDS) which induces fatal acquired immunodeficiency syndrome (AIDS) in 100% (25 of 25) of persistently viremic experimentally infected specific pathogen-free (SPF) cats after predictable survival periods ranging from <3 months (*acute immunodeficiency syndrome*) to > one year (*chronic immunodeficiency syndrome*), depending on the age of the cat at time of virus exposure. The pathogenesis of FeLV-FAIDS–induced feline immunodeficiency disease is characterized by: a prodromal period of largely asymptomatic viremia; progressive weight loss, lymphoid hyperplasia associated with viral replication in lymphoid follicles, lymphoid depletion associated with extinction of viral replication in lymphoid follicles, intractable diarrhea associated with necrosis of intestinal crypt epithelium, lymphopenia, suppressed lymphocyte blastogenesis, impaired cutaneous allograft rejection, hypogammaglobulinemia, and opportunistic infections such as bacterial respiratory disease and necrotizing stomatitis. The clinical onset of immunodeficiency syndrome correlates with the replication of a specific FeLV-FAIDS viral variant, detected principally as unintegrated viral DNA, in bone marrow, lymphoid tissues, and intestine. Two of seven cats with chronic immunodeficiency disease that survived >1 year after inoculation developed lymphoma affecting the marrow, intestine, spleen, and mesenteric nodes. Experimentally induced feline immunodeficiency syndrome, therefore, is a rapid and consistent in vivo model for prospective studies of the viral genetic determinants, pathogenesis, prevention, and therapy of retrovirus-induced immunodeficiency disease.

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THE HUMAN immunodeficiency virus (HIV)[1-7] has infected several million people worldwide, at least 50,000 of whom have contracted AIDS.[8-11] More than 36,000 cases of AIDS have been reported in the United States; >56% of these people, including 79% of those infected with HIV for ≥23 months, have died.[9,10] If 10% to 30% of the 1 to 1.5 million persons currently infected with HIV in the United States develop disease, 270,000 cases will occur by 1991,[10] the most serious epidemic of the past 50 years.[11]

Retrovirus-induced AIDS has long-standing precedent in feline leukemia virus (FeLV)-associated immunosuppression, a naturally occurring syndrome recognized for >15 years in cats infected with some isolates of FeLV.[12-18] The contagious transmission of FeLV was first recognized among dense urban animal populations of cats with high degrees of social contact and high incidences of leukemia and other infectious diseases.[18-19] It has subsequently become clear that many more cats die from the cytosuppressive consequences of FeLV infections (immunodeficiency syndrome and bone marrow aplasia) than from leukemia[12,16-22], a paradigm perceived by Gallo and colleagues[23,24] in seeking a contagious exogenous retrovirus as a cause of immunodeficiency disease. Although HIV and FeLV infections clearly represent distinct retrovirus/host relationships, both infections involve horizontal transmission requiring close contact and transfer of secretions, sequential stages in the progression of infection and disease,[27-29] virus strain-related variations in tissue tropism and pathogenicity,[29-35] and viral latency and activation.[36]

Whether retrovirus-associated immunodeficiency is determined principally by specific viral genotypes (variants) or whether, alternatively, disease is triggered principally by cofactors such as genetic background or frequent viral or other intercurrent infections is an issue important to the understanding of retrovirus pathogenesis. We provide evidence for the former tenet in describing the identification, transmission, and pathogenesis of a naturally occurring, intensely immunosuppressive isolate of feline leukemia virus (designated here as FeLV-FAIDS) which consistently induces lethal immunodeficiency syndrome, the onset of which is associated with replication of a disease-specific variant virus genome in bone marrow, lymphoid tissue, and intestine of experimentally infected animals.

MATERIALS AND METHODS

Animals

The specific pathogen-free (SPF) cats used in the transmission studies we report were from a breeding colony of cesarian-derived SPF cats maintained in the Department of Pathology, Colorado State University. These animals are free of infection with and immunity to horizontally transmitted feline viruses, including FeLV. Age-matched, noninoculated, noncontact control SPF cats were obtained from the same source, for which there is a substantial database of normal age-adjusted hematologic data.

Virus, Inoculation, and Sample Collection

The original FeLV-FAIDS inoculum consisted of a 20% (wt/vol) viable-cell-free extract of thymic lymphoma tissue from an FeLV-infected pet cat in which the lymphoma had regrown after regression induced by radiotherapy. In most inoculation experiments, virus was administered intraperitoneally (IP); in two series of inoculations, the

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intranasal route was used (both routes induced infection and disease) (Table 1). Inocula for serial passage experiments were prepared as 20% (wt/vol) extracts of bone marrow, spleen, or lymph node from animals that had developed fatal immunodeficiency disease in the first virus passage (Table 1). All inocula contained between 1 and 2×10^5 focus forming units (ffu) of infectious FeLV. One group of two weanling animals was inoculated with virus harvested from a feline fibroblast cell line inoculated with the original stock of FeLV-FAIDS. Likewise, FeLV-FAIDS was characterized morphologically by electronmicroscopic examination of glutaraldehyde-fixed, osmium tetroxide-postfixed inoculated feline fibroblast cell cultures.

Blood samples were collected from each inoculated cat prior to infection and at weekly intervals for the first 6 weeks postinoculation and biweekly thereafter. Bone marrow samples were collected at various intervals (most often biweekly) by needle aspiration from the femur or humerus of inoculated cats. For all inoculation, blood collection, and bone marrow sampling procedures, animals were anesthetized with ketamine hydrochloride.

Assays to Monitor FeLV-FAIDS Infection in Cats

Immunofluorescence for FeLV p27 group-specific antigen in blood cells and tissues. FeLV-FAIDS group-specific antigen (gag gene-coded major viral structural protein p27) was detected by indirect immunofluorescence staining of methanol-fixed blood films[32-37] and on methanol-fixed paraffin-embedded tissues.[38] The goat anti-FeLV p27 antiserum used was extensively absorbed with feline blood and bone marrow cells to eliminate non-specific reactivity due to natural anti-feline tissue antibody in goat or rabbit sera.[32,37] The persistent presence of FeLV p27 antigen in blood cells (neutrophils, platelets, lymphocytes) correlates >95% of all instances with the presence of infectious virus in blood.[39,40]

Enzyme-linked immunosorbent assay (ELISA) for FeLV antibody. FeLV antibody was determined by an ELISA using sucrose density-gradient purified whole FeLV (KT isolated from the FL74 lymphoblast cell line) as antigen, feline test serum, and peroxidase-conjugated goat-anti-feline IgG as the secondary reagent (Miles Scientific, Naperville, IL).[41]

Quantitation of infectious virus in inocula. Infectious FeLV in tissue extracts was determined by the clone 81 focus induction assay described by Fischinger et al[42] Particle-associated reverse transcriptase was also demonstrated as described for FeLV by Nicolson et al.[43]

Detection of viral DNA in tissues. FeLV-FAIDS integrated and unintegrated viral DNA was detected in tissues of infected cats by Southern blotting[44] using Kpnl endonuclease digestion and hybridization to an exogenous FeLV-specific DNA probe that identifies a 250-base pair (bp) sequence in the unique region of the long terminal repeat of exogenous feline retroviruses only, as detailed by Mullins et al.[45,46] In brief, total cellular DNA (7.5 μg) was purified, digested with Kpnl, electrophoresed in 1.2% agarose gels, transferred to nitrocellulose, hybridized with the exogenous FeLV-specific probe (exU3) with 10% dextran sulphate and 50% formamide, washed, then exposed to x-ray film as described previously.[45-47]

Hematologic assays. Complete blood cell counts were performed at biweekly intervals (Coulter S-plus calibrated for feline blood). In some cats with terminal immunodeficiency disease, bone marrow cell clonogenic colony-forming assays of erythroid and granulocyte/macrophage progenitor cells were performed in methylcellulose semisolid medium using techniques described by Abkowitz et al[48] and modified by Gasper et al[49] Albumin and globulin classes in terminal sera from some cats were determined by electrophoresis using standard procedures.

Lymphocyte Blastogenesis and Skin Grafting

Mitogen-driven, feline T lymphocyte blastogenesis was determined as previously described originally by Cockerell et al[50] and extended by Rojko et al[51] for feline blood lymphocytes. Blastogenesis assays were performed by incubating Ficoll-metrizoite-separated blood mononuclear cells with either concanavalin A (1 μg/ 2×10^5 cells, a feline T cell mitogen)[51]; staphylococcus protein A (20 formalin-fixed bacteria per cell, a feline T and B cell mitogen)[51]; or pokeweed mitogen (10 μg/ 2×10^5 cells, a feline T and B cell mitogen).[51] Net cell DNA-associated uptake of ^{3}H thymidine in cells pulse-labeled for 18 hours before harvest with 0.5 μCi of ^{3}H thymidine/ 2×10^5 cells was then quantitated by liquid scintillation. Allogeneic cutaneous allographs (1.5 cm^2) from a single unrelated donor cat were applied and rejection intervals were assessed as described previously in SPF cats by Perryman et al.[14]

Histologic Examination

Necropsies were performed and macroscopic lesions were recorded for all cats that died or were killed due to symptoms of severe immunodeficiency disease. Duplicate tissue sections were collected in absolute methanol for immunofluorescence and in either formalin or Bouin's fixative for conventional histologic section preparation. The following tissues from each of 20 animals were examined histopathologically; mandibular, pharyngeal, mesenteric, and colonic lymph nodes, spleen, bone marrow, thymus, liver, kidney, adrenal, lung, heart, and small intestine (duodenum, jejunum, ileum) colon, urinary bladder, trachea, esophagus, thyroid, nostril, and brain.

RESULTS

Virus Isolation and Characteristics

The lymphocytopathic FeLV we describe was identified by intrathymic inoculation of newborn kittens with an extract of thymic lymphoma tissue from an FeLV-infected pet cat in which the lymphoma had regrown after regression induced by radiotherapy. Five of six inoculated kittens died with severe lymphoid depletion and enteritis within 2 months postinoculation (Table 1). The viral isolate (designated here as FeLV-FAIDS) was identified as a feline leukemia retrovirus[52] by the following criteria: type C morphology by electronmicroscopy; immunoreactive FeLV group-specific (gag) and envelope proteins as determined by immunofluorescence and immunoelectrophoresis, respectively; virus particle-associated reverse transcriptase activity[53,54]; and FeLV interference inducing activity characteristic of FeLV subgroup A and B viruses[55-58] (performed in the laboratory of Drs Oswald Jarrett and David Onions, University of Glasgow, Glasgow, Scotland).

Experimental Transmission and Disease Induction

To determine whether the lymphocytopathic activity of the FeLV-FAIDS isolate was restricted to or dependent on infection during the neonatal period, we inoculated groups of SPF cats aged 8, 15, and 24 weeks with the original virus stock. We then performed two serial virus subpassages in 8-week-old SPF cats using marrow, spleen, and lymph node inocula containing between 1 and 2×10^5 (infectious) ffu[42] of FeLV (Table 1).

Table 1. Experimental Transmission of FAIDS in Specific Pathogen-Free Cats

Age of Cats at Inoculation	Route of Inoculation	Virus Passage Number	Cats Inoculated (n)	Cats With Persistent Viremia (n)	Cats With FAIDS (n)	Survival Time of Viremic Cats (Days)	Cats With Lymphocytopenia (n)	Cats With Suppressed Lymphocyte Blastogenesis (n)	Cats With Prolonged Skin Graft Rejection (n)	Clinical Signs	Lesions
1 Day	Intrathymic	I	6	5	5	59 ± 3	5	ND	ND	Weight loss, diarrhea	Severe lymphoid depletion, necrotizing enterocolitis
8 Weeks	Intraperitoneal	I, II, III	13	11	11	99 ± 59	11	5/5*	2/2†	Weight loss, thrush, diarrhea	Lymphoid hyperplasia, severe lymphoid depletion, necrotizing enterocolitis, stomatitis
15 Weeks	Oronasal	I	10	6	6	241 ± 166	6	2/2	ND	Diarrhea, severe chronic weight loss	Lymphoid hyperplasia, severe lymphoid depletion
24 Weeks	Oronasal	I	6	3	3	451 ± 84	3	3/3	2/3	Severe chronic weight loss, diarrhea, anemia	Lymphoid depletion, enterocolitis
Totals			35	25	25	—	25	10/10	4/5		

Cats were inoculated with between 1 and 2 × 10^5 ffu FeLV-FAIDS as determined by clone 81 assay.[42]

*Number affected/total tested.

†Prolonged rejection = >16 days ($p < 0.05$ by Student's *t* test) (mean for normal SPF cats 13.1 = +1.5 days, n = 20). A single unrelated donor was used for all cats.

Twenty-five of 35 cats of various ages (71%) inoculated with FeLV-FAIDS developed persistent viremia within 2 to 4 weeks after inoculation. All the viremic cats (25 of 25) died of immunodeficiency disease from 25 to 524 days after inoculation (mean 171 ± 156 days) (Table 1). None of 10 cats that developed transient, regressive infection after virus inoculation developed any sign of disease after observation periods as long as 600 days. ELISA antibody titers against FeLV-FAIDS developed in both progressively and regressively infected cats; however, titers of 25 viremic cats destined to develop immunodeficiency syndrome declined to minimal levels (geometric mean 1:4.3 ± 3.6) 3 to >40 weeks before clinical disease developed. The terminal mean viral antibody titer of ten regressively infected cats was 1:58 ± 5.6.

The susceptibility of SPF cats to induction of persistent FeLV-FAIDS viremia, the latent period for clinical immunodeficiency disease induction, and the incubation period for disease induction all were related to age of cat at time of inoculation (Table 1); 85% of animals <8 weeks old at inoculation developed persistent viremia and survived for an average of 80 ± 58 days postinoculation (DPI). In contrast, 56% of cats >15 weeks old at inoculation developed persistent viremia and survived for an average of 311 ± 173 DPI. Therefore, relative to other characterized FeLV isolates so studied,[30] FeLV-FAIDS exhibited considerable virulence and was capable of producing persistent viremia and disease in young adult cats after a single intranasal or intraperitoneal inoculation and without the concurrent administration of adrenal corticosteroids,[59] a cofactor required to induce >50% incidence of progressive infection in adult cats with many FeLV isolates.[30]

Clinical Courses of Disease

Two clinical courses of feline immunodeficiency syndrome were recognized. The first clinical course, acute or early onset immunodeficiency disease, was characterized by a survival period of <180 DPI (mean 86 ± 42; range 25 to 160) and developed in 72% (18 of 25) of all viremic cats and 94% (15 of 16) of viremic cats that were 8 weeks or less at inoculation. A short (2 to 10 weeks) asymptomatic period was followed by progressive lymphocytopenia, suppressed lymphocyte blastogenesis, failure to gain weight and/or progressive loss of weight leading to emaciation (Fig 1A), persistent diarrhea, and opportunistic infections including bacterial rhinitis, pneumonia, and necrotizing stomatitis (Fig 1B).

The second clinical course, chronic or late-onset feline AIDS, was characterized by a survival period of >180 DPI (mean 396 ± 113, 4.6 times that of cats with acute AIDS) and occurred in 28% (7 of 25) of all viremic cats but in 67% (6 of 9) of cats aged ≥15 weeks at inoculation. The chronic disease course was marked by a relatively long (6 months to >1 year) and largely asymptomatic period of viremia, subnormal weight gain, and progressive lymphocytopenia. This prodromal period was followed by an accelerated symptomatic phase characterized by relentless diarrhea and accelerated weight loss leading to terminal debilitation. Thus, the chronic clinical course of experimental feline AIDS more closely simulated the protracted natural evolution of most cases in immunodeficiency disease in humans.[8]

Hematologic and Immunologic Features of FeLV-FAIDS Infection

The mean lymphocyte count for all symptomatic and presymptomatic FeLV-FAIDS inoculated cats over the course of all sampling periods postinoculation (2 to 67 weeks) was significantly below that of age-matched controls (Fig 2, Table 2). Lymphocyte mitogen-driven blastogenesis characteristically was depressed in symptomatic cats as compared with uninoculated controls (Figs 2 and 3 and Table 2). Skin allograft rejection time was significantly prolonged in four of five symptomatic FeLV-FAIDS–infected cats *v* normal controls (Table 2). Gamma-globulin levels in terminal sera from ten AIDS cats assayed were 44% of those of controls (Table 2). Thus, evidence for severe deficits in both T and B lymphocyte function were present in cats with immunodeficiency syndrome.

Nonlymphocytic hematologic deficits also were detected in FeLV-FAIDS–inoculated cats. Circulating granulocytes and monocytes in virus-inoculated cats were significantly less than controls over the course of all sampling periods (Table 2). Terminal hematocrits of inoculated cats also were less than controls and evidence of erythropoietic regeneration was absent (reticulocyte counts were <50,000 in infected cats *v* >150,000 in controls). Clonogenic colony-forming assays of bone marrow cells from each of four cats with immunodeficiency syndrome tested revealed suppression of erythroid progenitor cells without discernible impairment of myeloid progenitor cell growth (Table 2).

Lesions

A consistent array of lesions was present in cats with acute feline AIDS. These were: (a) thymic atrophy in all (13 of 13) cats examined histologically (thymic remnants were detectable only by microscopy); (b) lymphoid depletion involving follicular and parafollicular lymphocytes in lymph nodes, spleen, and Peyer's patches, resulting in ablation of entire follicles (9 of 13 animals) (Fig 1B and D); (c) follicular lymphoid hyperplasia in some cats (6 of 13) characterized by large, sometimes irregularly shaped follicles with prominent germinal centers comprised of lymphoblasts (Fig 1C); and (4) necrotizing enterocolitis (13 of 13) marked by necrosis and partial regeneration of the mucosal germinal epithelium, neutrophil and macrophage infiltration, and atrophy of mucosal villi (Fig 1F). The histopathogenesis of the lymphoid lesions in FeLV-FAIDS–infected cats, therefore, resembles that in human AIDS, ie, an apparent sequence of follicular lymphoid hyperplasia of variable duration followed by progressive follicular ablation and ultimately extensive parafollicular and medullary lymphoid depletion.[60-63]

Lesions of lymphoid depletion in cats with chronic course of disease were more pronounced than in animals with the acute syndrome. Bone marrow cellularity in terminal immunodeficiency cats was significantly reduced *v* uninfected, age-matched SPF control cats. Four of seven animals with chronic disease that survived >400 DPI developed terminal

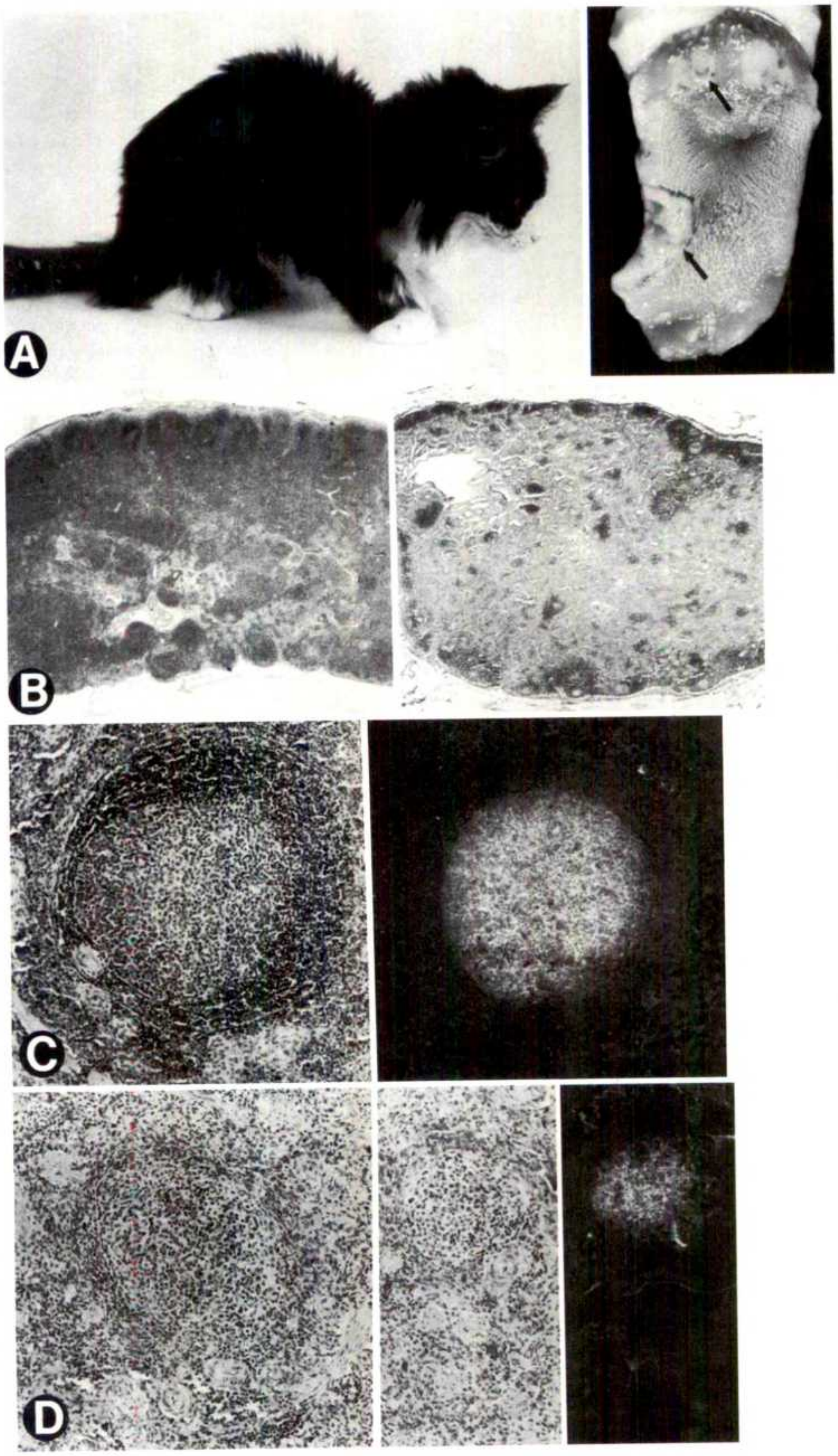

Fig 1. Pathogenesis of experimentally induced feline immunodeficiency disease. (A) Clinical appearance of a cat with advanced AIDS; chronic diarrhea, severe weight loss and debilitation are typical (left); opportunistic infection: necrotizing oral lesions (arrows) involving the tongue (right). (B) Normal lymph node from a control SPF cat; dense cortical and paracortical populations of lymphocytes are apparent (left). Comparable lymph node from a cat with terminal immunodeficiency syndrome; extensive depletion of lymphocytes in the cortical (follicular), paracortical, and medullary areas is evident (right). (C) Prodromal follicular lymphoid hyperplasia in a formalin-fixed, conventionally (H&E)-stained section of spleen from a cat infected with FeLV-FAIDS (left). FeLV-FAIDS replication in splenic follicle from the cat at left demonstrated by immunofluorescence in a methanol-fixed tissue section (right). (D) FeLV-FAIDS–induced lymphoid depletion, severely depleted splenic follicles in hematoxylin and eosin (H&E)-stained histologic sections (left and middle); corresponding immunofluorescence section (right) demonstrates concomitant depletion in viral replication.

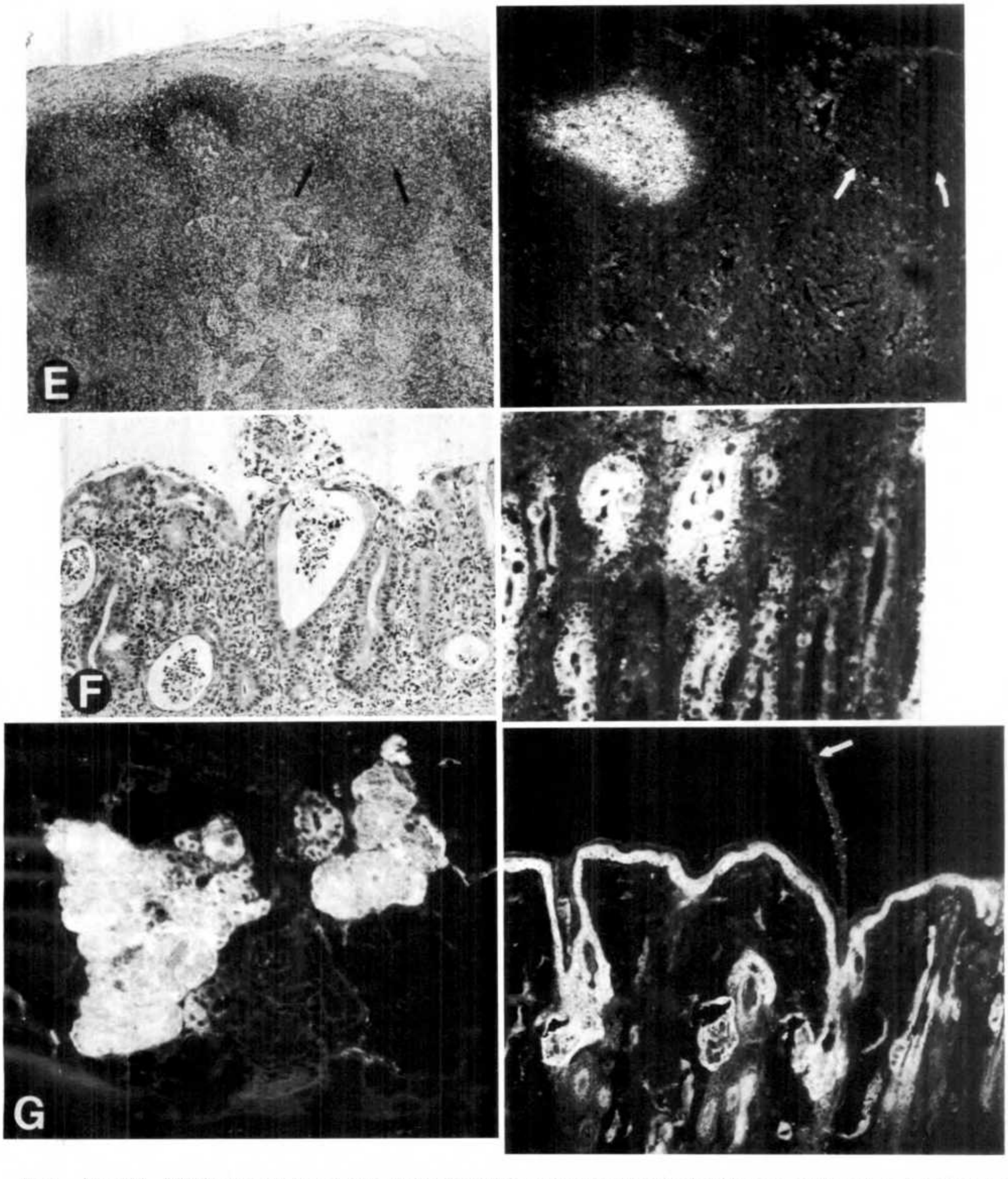

Fig 1. (Cont'd.) (E) Ablation of the cortical lymphoid follicles (left, arrows) correlated with extinction of viral replication in the same areas (right, arrows), whereas viral replication remained evident in the adjacent nondepleted follicle. (F) Necrotizing enteritis induced by FeLV-FAIDS, with depletion and regeneration of germinal epithelium in the colon (H&E section, left); replication of FeLV in colonic germinal epithelium of (immunofluorescence, right). (G) Replication of feline AIDS virus in salivary gland epithelium (left) and in the epidermis, hair follicles, and sebaceous glands of the nostril of an infected cat (right). Arrowhead = hair shaft (immunofluorescence as above).

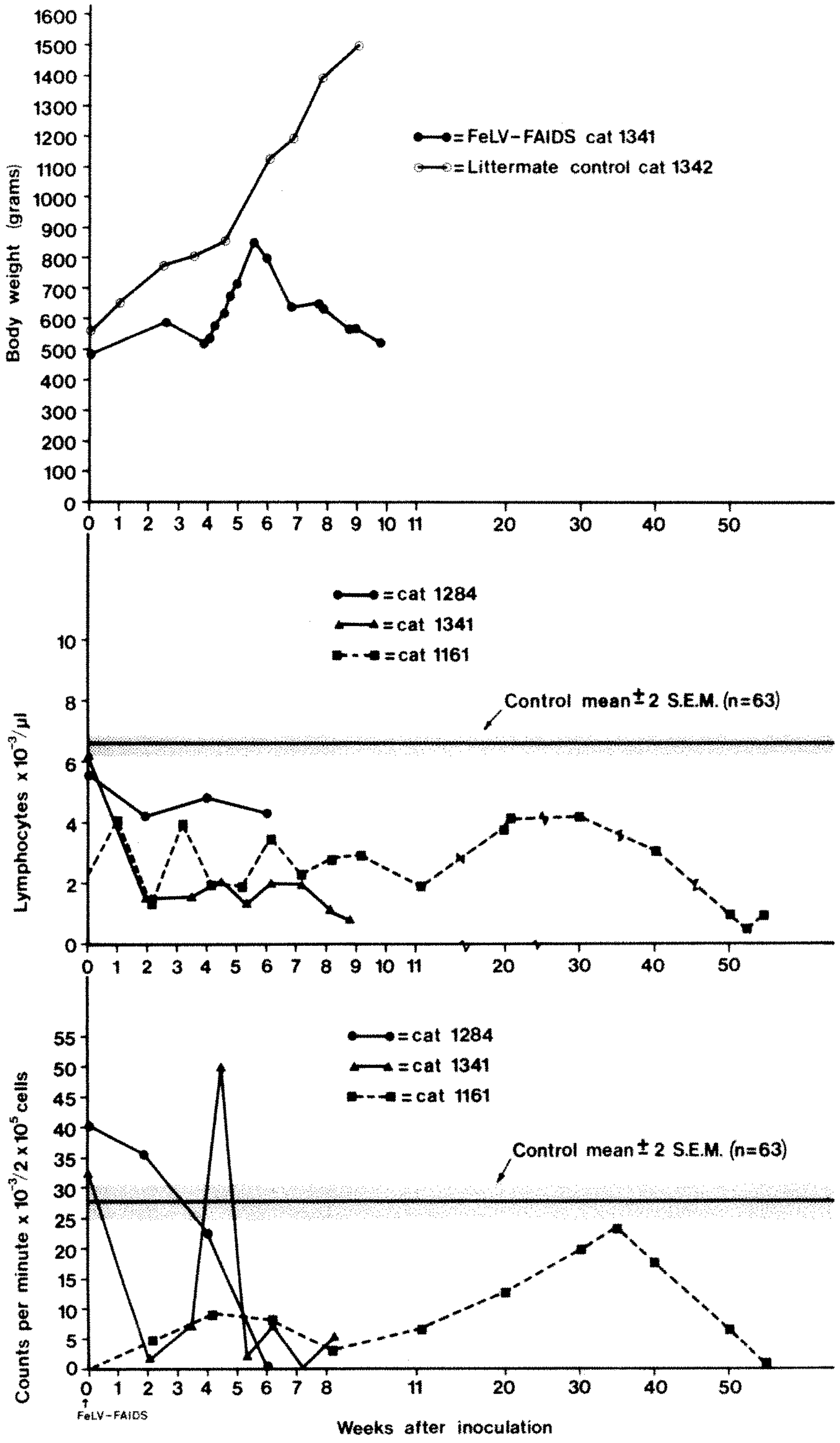

Fig 2. Sequential changes in body weight (top), lymphocyte numbers (middle), and lymphocyte mitogenic response to *Staphylococcus* protein A (bottom) in three cats inoculated with FeLV-FAIDS and representing the *acute* (cats 1284 and 1341) and *chronic* (cat 1161) courses of feline immunodeficiency disease.

lymphoblastic lymphoma superimposed on lymphoid depletion and affecting the marrow, intestine, and mesenteric nodes (see below).[64,65]

Virus Tissue Tropism

Immunofluorescence analysis of lymph node sections from cats inoculated with FeLV-FAIDS revealed intense viral replication (FeLV p27 gag protein) in nodes with many hyperplastic follicles but minimal viral replication in nodes with depleted follicles (Fig 1C through E). The extinction of viral replication in lymphoid tissues, therefore, correlated with extinction of lymphoid follicles. Viral replication in intestinal crypt epithelium also was correlated with evidence of antecedent necrosis of crypt epithelium (Fig 1F).

FeLV-FAIDS replication not associated with discernible cytopathic effect was detected in several cell types: ie,

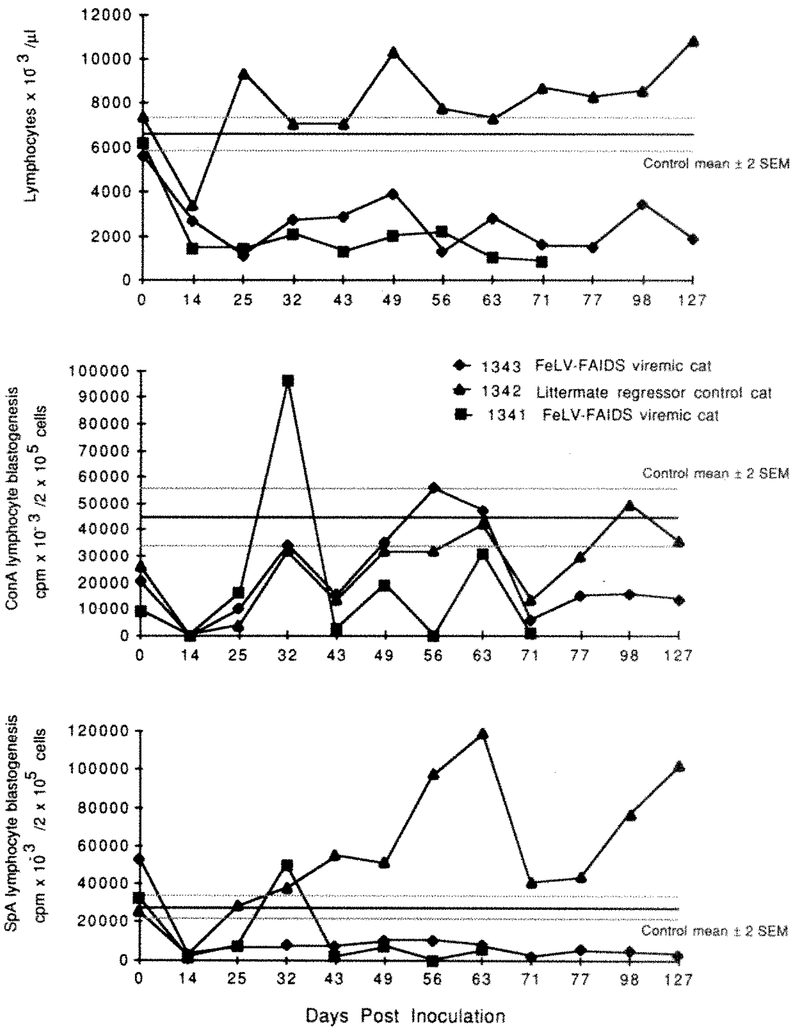

Fig 3. Sequential changes in blood lymphocyte numbers and blastogenic responses from representative cats inoculated with the mitogens concanavalin A and *Staphylococcus* protein A in representative FeLV-FAIDS–inoculated SPF cats that developed either persistent viremia (1341) or transient infection and regressive infection (1342) as compared with an uninoculated littermate control.

Table 2. Summary of Hematologic and Immunologic Data in Cats With Immunodeficiency Syndrome Induced by FeLV-FAIDS

Parameter Measured	FeLV-FAIDS Viremic Cats	Cats (n)	FeLV-FAIDS Regressors*	Cats (n)	Control SPF Cats	Cats (n)
Total lymphocytes/µL (all stages of infection)	3,486 ± 990	20	5,928 ± 213	8	6,579 ± 378	41
Total lymphocytes/µL (clinical disease)	1,092 ± 494	7				
Total granulocytes + monocytes (all stages of infection)	8,542 ± 483	20	6,520 ± 243	8	11,888 ± 990	41
Total granulocytes + monocytes (clinical disease)	7,820 ± 519	7				
Hematocrit (%) (all stages of infection)	32 ± 1	20	35 ± .3	8	33 ± 3.7	41
Hematocrit (%) (clinical disease)	21.3 ± 9.0	7				
Lymphocyte blastogenesis						
ConA (all stages of infection,	24,758 ± 3,410	20	33,396 ± 4,447	8	44,822 ± 5,411	41
ConA (clinical disease)	3,695 ± 1,464	8				
SpA (all stages of infection,	12,188 ± 3,011	20	29,328 ± 4,321	8	27,473 ± 2,967	41
SpA (clinical disease)	1,964 ± 1,043	8				
PWM (all stages of infection,	6,933 ± 905	20	11,880 ± 1,464	8	12,990 ± 1,459	41
PWM (clinical disease)	1,709 ± 827	8				
Total γ-globulin (mg/dL)	0.70 ± 0.9	—	ND		1.58 ± 0.32	
Skin allograft rejection (days)	18.5 ± 2.4	—	ND		13.1 ± 1.5	
Marrow CFU-e (no. of CFU/10(5) cells seeded)*	12 ± 1.7†	—	ND		134 ± 2	
Marrow BFU-e (no. of CFU/10(5) cells seeded)	14 ± 9†	—	ND		82 ± 5	
Marrow CFU-GM (no. of CFU/10(5) cells seeded)	38 ± 8†	—	ND		37 ± 1	

*No disease developed after observation periods up to 600 days postinoculation.

†Hematopoietic colony-forming units–erythroid (CFU-e, BFU-e) or granulocyte macrophage (CFU-GM) generated by feline bone marrow cells assayed in a methylcellulose medium culture system.[80] For FAIDS cats, n = 4; for control cats, n = 320.

circulating neutrophils and platelets, salivary gland acinar and duct epithelium, mucosal epithelium of the esophagus, nasal passages, and bronchioles, and the glandular and hair follicle epithelium of the nostrils (Fig 1g). Neither viral antigen nor lesions were found in the testes, ovary, uterus, kidney, liver, adrenal, thyroid, heart, or brain. Thus, the feline AIDS-inducing virus replicated in a range of hemolymphatic and epithelial tissues but appeared to be cytopathic only in bone marrow, intestine, and lymphoid tissue.

The intestinal lesions in cats infected with FeLV-FAIDS resembled those produced by the feline and canine parvoviruses. Therefore, we assayed both original viral inocula and intestinal mucosal extracts from three cats with fatal immunodeficiency syndrome for feline parvoviral antigen by an ELISA sensitive to 2 pg viral antigen. Each was negative. Moreover, six of six SPF cats immunized with commercial feline panleukopenia parvovirus vaccine developed immunodeficiency disease featuring chronic diarrhea and enteritis after FeLV-FAIDS inoculation.

Southern Blot Analysis of Viral DNA in Tissues

Southern blot analyses of purified cellular DNA demonstrated viral DNA in all (90 of 90) virus-antigen–positive tissue and bone marrow samples from all 25 viremic cats examined (Table 1). Highest levels of FeLV-FAIDS DNA were in bone marrow, intestine, and lymphoid tissues. As initially reported,[47] two significant viral genomes were detected in the tissues of infected cats: (a) a ubiquitous "common" or "early" form virus genome recognized as a 3.65-kb internal viral DNA fragment (Fig 4) in all virus-antigen–positive tissues of all viremic asymptomatic and symptomatic cats examined at all postinoculation intervals; and (b) a variant genome (designated variant A) characterized by an additional KpnI site in the extracellular glycoprotein gene (gp70) region of the viral envelope gene and generating a signature 2.1-kb 3′ KpnI fragment (variant A), which was detected in large amounts (15 to 50 copies/cell), predominately as unintegrated viral DNA (Fig 4) in the bone marrow, intestine, and nonseverely-depleted lymphoid tissues only of animals with symptomatic immunodeficiency syndrome. In tissues of animals with severe lymphoid depletion, minimal amounts of viral DNA were discernible by Southern blotting and minimal viral antigen was demonstrable by immunofluorescence (Fig 1C through E). Thus, FeLV-FAIDS common-form virus was demonstrated in all viremic cats both before and after the onset of disease, whereas the appearance and amplification of variant A virus genome in marrow, intestine, and lymphoid tissues was correlated strongly with the development of symptomatic, ultimately fatal immunodeficiency disease.

Late-Onset Neoplasia in Some Animals Infected With FeLV-FAIDS

Two of the seven animals that developed chronic immunodeficiency syndrome, both of which survived for >400 days after inoculation (471 and 524 days, respectively), also developed terminal T cell or null cell lymphoma affecting the bone marrow, small intestine, and to a lesser degree, mesenteric lymph nodes. Histologic examination of the mesenteric lymph nodes indicated that proliferation of neoplastic lymphoblasts was superimposed on lymphoid involution. Other nodes contained only the lymphoid depletion typical of advanced immunodeficiency syndrome. Southern blot analysis of lymphoma tissues revealed clonal viral integration

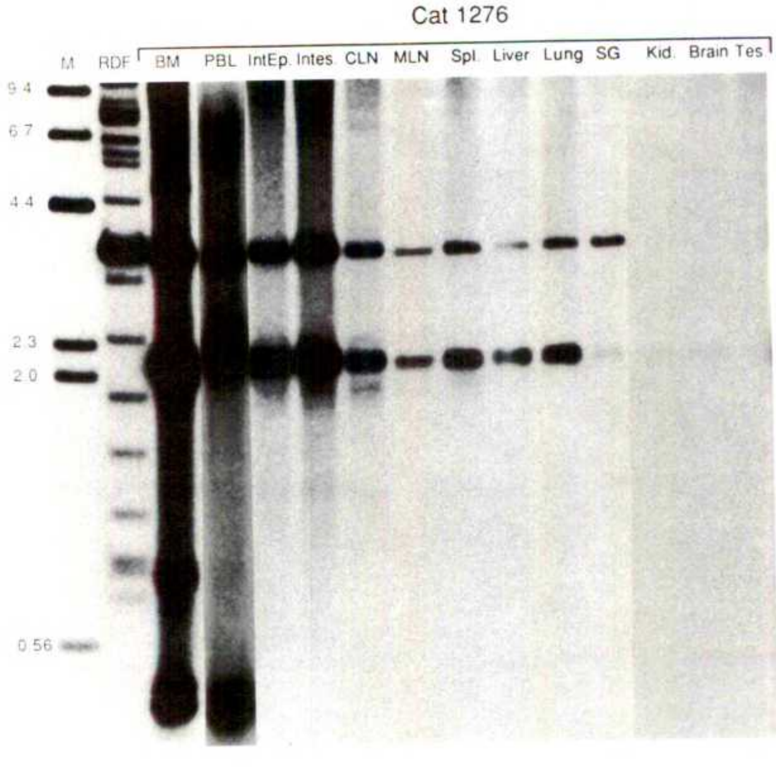

Fig 4. Tissue distribution of FeLV-FAIDS DNA in an animal with acute feline immunodeficiency syndrome; 7.5 μg total cellular DNA was purified from frozen postmortem samples of the tissues indicated and digested with the restriction endonuclease KpnI. M, mol wt markers (kb scale); RDF, a cloned cell line infected with FeLV-B-GA[43]; BM, bone marrow; PBL, peripheral blood lymphocytes; IntEp., intestinal epithelium, Intes., intestine; CLN and MLN, colonic and mesenteric lymph nodes, respectively; Spl., spleen; SG, salivary gland; Kid., kidney; Tes., testis. The FeLV-FAIDS common form (CF) internal virus band migrates at 3.65 kb, whereas the AIDS-specific variant A (VA) band migrates at 2.1 kb. The bands at 0.35 and 0.45 kb in the BM and PBL lanes correspond to 5′-LTR sequences detected as a consequence of the presence of unintegrated DNA, derived from linear and circular DNA, respectively.

patterns confirming the presence of a clonal proliferation of virus-genome–bearing cells (data not shown). Thus, prolonged FeLV-FAIDS viremia and/or chronic immunosuppression may result in long-latency lymphomagenesis, as has been observed in some HIV-infected patients.[66-68]

DISCUSSION

Although a lymphoid depletion/immunodeficiency syndrome has been recognized for >15 years in cats infected with feline leukemia virus,[12-18] the disease has neither been consistently reproduced with FeLV inocula nor has its relationship to virus genotype *v* other cofactors been elucidated. Cats infected with the most ubiquitous naturally occurring feline leukemia viruses—interference subgroup A (FeLV-A)[55-58]—commonly remain persistently viremic and asymptomatic for long periods. Viruses of subgroups B or C occur naturally only in combination with FeLV-A[57-58] and probably have evolved from the FeLV-A either through mutation or through recombination with silent multicopy endogenous FeLV sequences in the feline genome (Mullins JI et al, manuscript in preparation, and refs 52 and 58). It is plausible, therefore, that the acute pathogenicity associated with some FeLV field isolates reflects the presence of cytopathic virus variants in these inocula. Further definition of the molecular and biologic properties of FeLV variant genomes should aid in elucidating the mechanisms of disease induction by feline leukemia viruses. The studies we report define the biologic and virologic properties of a naturally occurring intensely immunosuppressive isolate of FeLV which consistently induces fatal immunodeficiency syndrome after incubation periods as short as 3 months to as long as 43 months, an interval governed principally by the age of the cat at time of infection. The onset of clinical immunodeficiency syndrome coincides with the appearance of a characteristic variant viral genome which replicates in bone marrow, lymphoid tissues, and intestine, predominantly as unintegrated viral DNA (UVD). The association of unintegrated retroviral replication with cytopathicity has been demonstrated for reticuloendotheliosis virus,[69] visna virus,[70] avian osteopetrosis virus,[71] and HIV.[33] In FeLV-FAIDS–associated immunodeficiency disease, the replication of UVD in target tissues is notable in its magnitude, persistence, and correlation with disease onset and lesions in vivo.

The consistent experimental induction of immunodeficiency disease by a specific type C retrovirus isolate in outbred animals free of intercurrent infection with other pathogens supports the tenet that viral genome rather than host or environmental factors plays the major role in induction of immunodeficiency disease. These results also suggest that neither D type morphology nor a complement of supranumery genes is essential for induction of immunodeficiency syndrome by retroviruses. Our continuing work with FeLV-FAIDS and another feline leukemia virus variant that induces aplastic anemia (FeLV-C-Sarma) suggests that drastic differences in target cell specificity, cytopathic activity, and pathogenicity of FeLVs in vivo are principally the consequence of subtle alterations in the extracellular glycoprotein gene (Riedel NO, manuscripts in preparation).[72] Thus, retrovirus glycoprotein gene variants that arise in certain cells, tissues, or stages of infection and possess altered cell tropism and pathogenicity may be responsible for induction of immunodeficiency or other disease syndromes.[33,34,73,74,77,78]

Both Southern blotting and immunofluorescence results indicate that the principal targets for FeLV-FAIDS variant replication and cytopathic effect (ie, bone marrow, intestine, and lymphoid tissues) are concordant. Apparently, noncytopathic viral replication was detected in several epithelial tissues important to viral excretion and contagious transfer (eg, mucosal epithelium of the pharynx, esophagus, nostrils, salivary gland), as has been demonstrated previously for the Rickard isolate of feline leukemia virus.[27] The causal relationship, if any, between unintegrated retroviral DNA replication and host cell death, originally identified by Keshet and Temin[69] in cell cultures infected with avian reticuloendotheliosis virus, remains unclear. The presence of UVD per se may not be directly responsible for cytotoxicity, since UVD in the absence of cell degeneration has been observed in HIV-infected T cell lines[78] and in the proliferative bone lesions of avian osteopetrosis.[71] The unintegrated replication of an immunodeficiency disease-associated FeLV genome in target tissues of infected cats does, however, provide an additional stimulus for study of the relationship between retroviral nonintegrative replication and genesis of cytopathic disease.

The correlation among FeLV-FAIDS replication in intestinal germinal epithelium, persistent diarrhea, and necrosis of mucosal epithelium detected in infected cats suggests that intestinal tropism may be a primary rather than exclusively a secondary manifestation of immunodeficiency disease-inducing retroviruses. The association of AIDS with enteropathy, chronic diarrhea, and intractable weight loss, now recognized as the chief presenting symptom characteristic of African AIDS,[11] has been considered secondary to opportunistic infections associated with immunosuppression (eg, cryptosporidiosis). In SPF cats with FeLV-FAIDS–associated enteritis, unlike the situation in naturally infected people or cats with immunodeficiency syndrome, neither pathogenic bacteria, viruses, nor parasites are associated with or required for development of the intestinal lesions. Although the necrosis and regeneration of the intestinal crypt epithelium in FeLV-FAIDS–infected cats resembles that caused by feline parvovirus (an indigenous feline virus which also infects mitotically active cells and could potentially be responsible for enteritis in cats naturally infected with FeLV), our studies indicate that the feline parvovirus is not involved in FeLV-FAIDS–associated enteropathy. In that intestinal crypt cell degeneration also has been associated with the enteropathy affecting many AIDS patients,[64] further consideration should be given to intestinal epithelium as a target tissue for cytopathic retroviruses.

FeLV-associated immunodeficiency syndrome, therefore, can be rapidly and consistently transmitted experimentally with a naturally occurring isolate of feline leukemia virus. The course of feline AIDS varies with and can be manipulated by the age of the animal at time of infection. Our results further suggest that the onset of clinical disease is associated with the replication, first in bone marrow and then in lymphoid tissues and intestine, of a disease-specific viral variant, the accelerated cytopathic replication of which is responsible for precipitation of immunodeficiency disease. It seems probable that such immunosuppressive FeLV variants are generated continually in nature and that previous experimental studies in which AIDS-like syndromes have been induced in cats may have involved inocula containing similar lymphocytopathic virus genomes. It also seems plausible that similar mechanisms involving more and less pathogenic, and possibly defective, variants of the human AIDS retroviruses are involved in onset and progression of human AIDS.

Since this article was submitted, Pedersen et al[79] have reported the isolation of a feline lenti-like virus (designated FTLV) from a group of cats with multiple chronic infections suggestive of underlying immunosuppression syndrome. This newly recognized non-FeLV feline retrovirus, which contains a Mg^{2+}-dependent reverse transcriptase, is of type D morphology, has been propagated only in freshly isolated, mitogen-pulsed feline lymphocytes, and is distinct from the pathogenic FeLV isolate we describe. FeLV-FAIDS transcriptase strongly prefers Mn++ catalysis, is of C type morphology, replicates efficiently in fibroblasts as well as lymphocytes, contains characteristic FeLV gag and env proteins, and hybridizes with a DNA probe specific for exogenous FeLV LTR U3-sequences.[47] Cats inoculated with the original FeLV-FAIDS inoculum, subpassaged virus, and normal SPF cats are negative for FTLV antibody by immunofluorescence and Western blotting assays (performed in the laboratory of Dr Niels C. Pedersen). Neither the original FeLV-FAIDS inoculum nor inoculated lymphocyte or fibroblast cultures generate Mg^{2+}-dependent reverse transcriptase activity. Moreover, the full-length genomes of the FeLV-FAIDS common form and variant A viruses described here have recently been molecularly cloned (Overbaugh JM et al, manuscript in preparation) and the variant virus shown to be capable of producing both lymphocyte killing in vitro and fatal immunodeficiency syndrome in vivo. Characterization of the pathogenic determinants of this immunodeficiency-inducing feline leukemia virus should contribute significantly to the understanding of retrovirus-mediated immunodeficiency diseases.

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Interleukin 2-Activated Killer Cells in Patients Following Transplants of Soybean Lectin-Separated and E Rosette-Depleted Bone Marrow

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During the early period following bone marrow transplantation before the immune system has reached full functional maturity, unprimed, nonspecific lytic systems may play a critical role as antiviral or antitumor effectors. The reconstitution of cells with this potential is of particular importance in recipients of bone marrow that has been depleted of mature T lymphocytes to prevent graft v host disease (GVHD). We examined the recovery of natural killer (NK) cells and interleukin 2 (IL 2)-augmented lymphokine-activated killer cells (LAK) in 48 patients at various intervals following transplantation of bone marrow depleted of mature cellular elements by treatment with soybean agglutinin and sheep RBCs (SBA$^-$E$^-$ BMT). We found normal levels of both NK and LAK activity as early as 3 weeks following SBA$^-$E$^-$ BMT. When compared with cells from controls, NK and LAK precursors from transplant recipients appeared to be activated in vivo in that freshly isolated peripheral blood mononuclear cells (PBMCs) from patients had an elevated cytolytic activity toward NK-insensitive targets and a more rapid response to activation by IL 2. In patients as well as controls, both LAK precursors and LAK effectors lacked antigens present on mature T lymphocytes (CD3, CD4, or CD8) but expressed antigens present on NK cells (CD2, CD16, and NKH1$_A$). The LAK cells did not lyse either donor or host peripheral blood T cell targets. The activity of NK effectors but not LAK precursors survived the in vivo total body irradiation used for pretransplant conditioning in three patients studied. LAK precursors could be demonstrated as early as 18 days following transplant at a time when the bone marrow contained primarily donor-derived cells. Little or no LAK activity could be generated from cells of the SBA$^-$E$^-$ BM graft itself, suggesting that LAK precursors differentiate rapidly from more primitive progenitors in the marrow graft. Thus, our data indicate that the NK and LAK lytic systems are among the earliest activities to recover during immune reconstitution following T cell-depleted BMTs.

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IN EXAMINING the basis of the immune deficiency present during the early period following bone marrow transplantation (BMT),[1-4] we and other researchers found that peripheral blood T cells in BMT patients differ from normal T cells in regard to a number of interleukin 2 (IL 2)-dependent functions.[5-11] The type and duration of the functional T cell deficits depends on whether the patient received conventional, unfractionated bone marrow containing mature T cells or marrow depleted of T cells by treatment with soybean agglutinin and E rosetting (SBA$^-$E$^-$ BMT). Initially, we observed in recipients of conventional grafts that circulating T cells produced little or no IL 2 when stimulated with phytohemagglutinin (PHA) or OKT3 during the first 18 months after BMT. In most of these patients peripheral blood mononuclear cell (PBMC) proliferative responses to OKT3 could be restored to nearly normal levels by the addition of IL 2 to the cultures.[7] In recipients of SBA$^-$E$^-$ BMT, the capacity of OKT3 to trigger T cell proliferation and IL 2 production was also deficient in the early posttransplant period.[8] Unlike responses in conventional BMT recipients, however, T cell proliferative responses to OKT3 in recipients of SBA$^-$E$^-$ BMT were only partially restored by exogenous IL 2. Moreover, during the first 8 weeks after BMT, most SBA$^-$E$^-$ BMT recipients had circulating T cells that spontaneously secreted IL 2, an activity not detected in patients receiving conventional grafts. These differences may reflect the absence of mature donor-derived IL 2-responsive cells in patients receiving SBA$^-$E$^-$ BMT.

In this study, we examined PBMCs from patients following SBA$^-$E$^-$ BMT for the regeneration of NK cells, and IL 2-inducible nonspecific cytotoxic cells (LAK). Our results demonstrate that unlike T cell functions, unprimed and IL 2-activated nonspecific cytolytic activities of PBMCs from patients receiving T cell-depleted grafts recover fully by week 3 posttransplant. Mature, IL 2-responsive cells appear not to have been transfused in the marrow inoculum, since these cells are removed during T cell depletion. These early IL 2-responsive cells share many of the phenotypic characteristics of NK cells.

MATERIALS AND METHODS

Patients and controls. Forty-eight patients were studied following transplantation with marrow from HLA-identical siblings that had been depleted of T cells by treatment with SBA and E rosetting based on the method of Reisner et al.[12] Included in the study group were 22 patients with chronic myelogenous leukemia (CML); 11 patients with acute myelogenous leukemia (AML) in first or second remission; 8 patients with acute lymphoblastic leukemia (ALL) in first, second, or third remission; 3 patients with non-Hodgkins lymphoma (NHL), and 2 patients with myelodysplastic syndrome (MYD).

Pretransplant conditioning was performed with cyclophosphamide (60 mg/kg/day × 2) and hyperfractionated total body irradiation (1,320 or 1,440 rad). Patients received no posttransplant

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prophylaxis against rejection or graft v host disease (GVHD). All patients studied achieved durable engraftment, as shown by cytogenetic analysis of bone marrow and peripheral blood leukocytes.

Most studies were performed at weekly intervals from week 3 to week 8 posttransplant. Twenty patients were studied at intervals up to 18 months posttransplant. Normal controls consisted of 34 laboratory personnel and two bone marrow donors.

Reagents. Human recombinant interleukin 2 (rIL 2) was kindly provided by the Cetus Corporation (Emeryville, CA). Monoclonal antibody (MoAb) to the IL 2 receptor Tac,[13] was a gift from Dr Thomas Waldmann (National Institutes of Health, Bethesda, MD). $NKH1_A$ was kindly provided by Dr J. Ritz (Harvard University, Boston). $NKH1_A$ is an IgM mouse MoAb raised against cloned NK cells[14] and reacts with the same 200-kd molecule recognized by Leu 19.[15] The OKT series of MoAbs were purchased from Ortho Diagnostics (Raritan, NJ) (OKT3, T11, T4, and T8), and the Leu 7 hybrid line was obtained from the ATLL (Bethesda, MD). Directly fluoresceinated MoAbs, HLE, mouse IgG, and Leu 11c as well as phycoerythrin-conjugated mouse IgG and unconjugated Leu 11b were purchased from Becton Dickinson (Mountain View, CA). Fluoresceinated Leu 11, T11, T3, and B1 and phycoerythrin-conjugated T3, DR, and NKH1 were purchased from Coulter Immunology (Hialeah, FL).

Cell preparations. PBMCs were separated from heparinized blood by centrifugation on Lymphoprep (Nyegaard, Oslo). The low-density cells were collected and washed twice with Ca^{2+}, Mg^{2+} free Hank's balanced salt solution (HBSS) and resuspended in RPMI 1640 supplemented with 25 mmol/L HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (RPMI). In some experiments, monocytes were depleted by adherence on plastic tissue culture dishes for 1 hour at 37°C. Monocyte-depleted PBMCs are referred to as peripheral blood lymphocytes (PBLs).

LAK and cytotoxic T lymphocytes cultures. For generation of LAK cells, PBMCs or PBLs were adjusted to a concentration of 1×10^6 cells/mL in RPMI containing 10% heat-inactivated human serum (HS) (M.A. Bioproducts, Walkersville, MD) and 100 U/mL rIL 2. No more than 20×10^6 cells were placed in 25-cm^2 tissue culture flasks and incubated from 2 to 9 days at 37°C in a humidified atmosphere of 5% CO_2. In one experiment, cytotoxic T lymphocytes (CTLs) were generated by coculture of 1×10^6 cells/mL PBLs from a normal control with an equal number of irradiated (3,000 rad), previously frozen PBLs from a bone marrow donor. The cells were cultured for 7 days in RPMI-10%HS supplemented with 100 U/mL rIL 2 as described above.

Cytotoxicity assays. The cytolytic activities of freshly isolated cells, CTLs, and LAK cells were measured in a standard ^{51}Cr release assay. The targets used were the NK-sensitive cell line K562 established from a patient with CML, the lymphoma cell line Daudi, and a cultured melanoma cell line SK-Mel-30 (kindly provided by Dr Alan Houghton, Memorial Sloan-Kettering Cancer Center, New York)[16] or PBMCs cultured for 72 hours with a 1:100 dilution of PHA-M (GIBCO, Grand Island, NY) (PHA blasts). The targets were labeled with 250 μCi Sodium ^{51}chromate (New England Nuclear, Boston) in 0.5 mL RPMI-10% FBS for 1 hour at 37°C. Following three washes, the targets were resuspended in RPMI-10% FBS at 5×10^4 cells/mL. Cultured effector cells were recovered from the flasks, washed twice in RPMI-10% FBS, and adjusted to yield effector to target cell (E:T) ratios from 100:1 to 0.5:1. Replicate cultures in a total volume of 0.2 mL were plated in 96-well round bottomed microtiter plates (Costar, Cambridge, MA), centrifuged at 1,000 rpm for 5 minutes, and incubated at 37°C for 4 hours. Spontaneous release was determined by incubating targets with 0.1 mL medium, and maximum ^{51}Cr release was determined by addition of 0.1 mL 5% Triton X-100 (New England Nuclear). At the end of the culture period, supernatants were harvested with a filter harvesting system (Skatron, Sterling, VA), and the filters were counted in a Beckman 5000 γ counter (Beckman Instruments, Irvine, CA). Data were expressed as either the percentage of specific lysis at a given E:T ratio as calculated from the equation: % specific lysis = [cpm experimental − CPM spontaneous)/(CPM maximum − CPM spontaneous)] × 100 or as lytic units, defined as the number of cells/10^6 resulting in specific lysis of 33.3% of 5,000 target cells.[17]

Cold-target competitions. Unlabeled targets were mixed with ^{51}Cr-labeled targets at ratios of unlabeled (cold) to labeled (hot) from 20:1 to 1:1 before addition to the microtiter plates. Effector cells were added at a constant E:T ratio (usually 10:1) to the various cold:hot (C:H) combinations, and the percentage of specific lysis was determined in the cytotoxicity assay described above. Results are expressed as the percentage of specific lysis at the indicated C:H ratios.

Immunofluorescence. Indirect and two-color direct immunofluorescence were performed using standard techniques.[18,19] Samples were analyzed on an EPICS C cell sorter (Coulter).

Treatment with Tac MoAb. Tac MoAb was added at the beginning of 3-day LAK cultures of PBLs from one normal control and two patients, at final culture concentrations ranging from 1:5,000 to 1:250 of purified ascites. Saturation of Tac binding sites was obtained with a dilution of 1:5,000 Tac as determined by indirect immunofluorescence (data not shown).

LAK precursor and effector depletion. PBLs from patients and normal controls were treated with MoAb plus complement or with complement alone prior to or following culture in rIL 2. Sodium azide containing MoAbs were dialyzed against PBS prior to use. PBLs were incubated for 60 minutes at 4°C with 0.1 mL/10^6 cells of 1:20 dilutions of OKT3, OKT11, OKT4, or OKT8, with a 1:125 dilution of $NKH1_A$, and with a 1:50 dilution of Leu 11b. The cells were centrifuged, and the MoAb was removed prior to addition of 0.5 mL of a 1:5 dilution of low-toxicity rabbit complement (Pel-Freez, Rogers, AR) and incubated at 25°C for 60 minutes. Cells treated prior to culture in rIL 2 were washed once with RPMI-10% FBS and then incubated for a second time with rabbit complement for 30 minutes at 25°C. The cells were washed twice in RPMI and adjusted for LAK culture or for cytotoxicity assay. LAK effector cells were counted and then adjusted to the same volume as those treated with complement alone prior to testing in the cytotoxicity assay. Freshly isolated PBLs treated with $NKH1_A$ or Leu 11b plus complement as described above had no lytic activity to K562 in 4-hour ^{51}Cr release assays (data not shown). PBLs that had been incubated overnight at 37°C to shed bound antibody were restained with directly FITC-conjugated OKT3, OKT11, OKT4, or OKT8 following antibody plus complement treatment and showed >97% depletion of the appropriate population.

RESULTS

NK cell activity following SBA^-E^- BMT. PBMCs were isolated from 30 patients 3 to 8 weeks following transplantation with HLA-matched bone marrow and tested for cytolytic activity to the NK-sensitive cell line K562. The results were compared with those from 34 normal control subjects tested simultaneously. The data shown in Table 1 demonstrate the early return of NK activity following SBA^-E^- BMT with a mean specific lysis of K562 of 46.3% in the patient group as compared with 48.1% for the controls. Both Daudi lymphoma cells and the melanoma cell line SK-Mel-30 were resistant to lysis by PBMCs from normal controls (1.9% ± 2.4% and 8.0% ± 8.1% specific lysis, respectively). There was low but greater lysis of both targets

Table 1. K Cell Activity Following SBA$^-$E$^-$ BMT

Target	Specific Lysis (%)* Normal Controls	No. Tested	Specific Lysis (%)* Patients Weeks 3-8	No. Tested	P Value†
K562	48.1 ± 18.8	34	46.3 ± 21.2	30	.60
Daudi	1.9 ± 2.4	7	16.9 ± 5.4	10	<.001
SK-Mel-30	8.0 ± 8.1	19	15.2 ± 15.4	13	.20

PBMCs from patients 3 to 8 weeks posttransplant and controls were tested in 4-hour ^{51}Cr release assays as described in the Materials and Methods section. Only the highest value was used for patients tested more than once during this interval.

*Results are expressed as the percentage of specific lysis ± SD at an effector/target ratio of 100:1.

†Mann-Whitney U test.

by PBMCs from the patients; the increased activity was significant only for Daudi (Mann-Whitney U test, $P < .001$). In one experiment, depletion of Leu 11$^+$ cells by antibody and complement removed the cytolytic activity of patient PBLs to both K562 and Daudi (38.2% and 25.8% specific lysis before treatment to 0.1% and 0.9%, respectively). Depletion with OKT3 plus complement had no effect on the killing of either target, thus indicating that both activities were mediated by T3$^-$ NK cells.

LAK activity following SBA$^-$E$^-$ BMT transplantation. In preliminary experiments with control PBLs, the addition of >100 U/mL of rIL 2 to LAK cultures did not increase lytic activity significantly. The results of three experiments, including two patients 8 weeks posttransplant are shown in Table 2. Although patient PBLs had somewhat more cytolytic activity with 250 U/mL of rIL 2, the difference was not great; culture with 500 U/mL appeared to decrease the activity slightly. Subsequent cultures of cells from both patient and controls were performed using 100 U/mL rIL 2. The results shown in Fig 1 are expressed as lytic units/10^6 PBMCs following 6 to 7 days of culture in IL 2. As early as week 3 following SBA$^-$E$^-$ BMT, the median LAK activity to all three targets, K562 (panel A), Daudi (panel B), and SK-Mel-30 (panel C) was not significantly different from the control group ($0.1 < P < .375$, Mann-Whitney U test). No significant change in the lytic activity was noted during the first 8 weeks post SBA$^-$E$^-$ BMT or in patients tested up to 76 weeks posttransplant. In none of 29 experiments was there lytic activity to any target following 3 to 7 days of culture under the same conditions in the absence of rIL 2 (data not shown).

Kinetics of LAK generation during culture in IL 2. The kinetics of the generation of LAK activity differed between patients and controls in that when assays were performed after 3 to 4 days of culture in rIL 2 (Table 3) there was significantly more lytic activity to both K562 and SK-Mel-30 by LAK cells from patients than by those from controls ($P < .05$). We performed LAK assays at 2-day intervals on PBMCs from 9 patients between 4 weeks and 5 months posttransplant, from 6 patients 6 to 18 months following transplantation, and from 12 normal controls. The data shown in Fig 2 are expressed as the percentage of the maximum lysis of K562 (panel A) or SK-Mel-30 (panel B) when tested at an E:T ratio of 10:1. Normal control PBMCs showed a plateau of maximal activity at days 5 to 6 to both targets. The mean percentage of specific lysis at day 6 of K562 was 71.5%±12.4% and of SK-Mel-30 was 64.0% ±19.3% on day 5. PBMCs from patients <5 months post SBA$^-$E$^-$ BMT had an earlier peak of activity; by day 4 the maximal percentage of specific lysis toward K562 (58.4% ± 27.4%) and SK-Mel-30 (58.2% ± 20.0%) was reached. By 5 months post-BMT, PBMCs from the patients required 6 days of culture with IL 2 to be maximally activated, similar to the normal control group.

Specificity of LAK cells. We examined the possibility that in addition to LAK cells, culture with rIL 2 may expand donor-derived or residual host CTL that may have autoreactivity[20] or that recognize minor histocompatibility antigens of either the host or the marrow donor.[21] LAK cells from five patients were tested for the ability to lyse 3-day phytohemagglutinin (PHA) blasts prepared from host or donor PBMCs frozen prior to transplantation. There was no lysis of the PHA blasts in any experiment (Table 4), although K562 and Daudi targets were readily lysed. In one experiment, donor-specific CTL generated from PBLs of a normal control could effectively lyse both donor and pretransplant host PHA blasts.

Cold-target competition experiments were performed on LAK cells from three patients <4 weeks posttransplant and two normal controls to determine if both NK-sensitive and NK-resistant targets were recognized by the same IL 2-

Table 2. Dose–Response of Patient and Control PBLs to Culture in rIL 2

UPN	Week Posttransplant	rIL 2 (U/mL)	Specific Lysis (%) K562 50:1	K562 25:1	K562 10:1	K562 1:1	Daudi or Mel-30 50:1	Daudi or Mel-30 25:1	Daudi or Mel-30 10:1	Daudi or Mel-30 1:1
Control		10	68.0	54.7	46.0	16.6	48.5	37.9	21.7	9.1
		100	80.0	74.9	75.2	65.6	92.3	82.9	66.7	53.4
		1,000	84.1	78.6	76.3	58.5	86.2	80.7	86.9	55.8
681	8	100	55.8	40.8	32.1	—	70.3	62.9	42.6	—
		250	59.8	42.3	25.6	—	80.6	69.1	45.0	—
		500	47.8	27.7	14.1	—	77.0	57.6	40.6	—
652	8	100	21.3	11.3	6.3	—	34.0	19.0	8.7	—
		250	39.4	15.4	7.9	—	36.0	18.7	10.8	—
		500	28.6	12.1	6.6	—	35.8	6.4	2.1	—

PBLs were cultured 6 days (control) or 3 days (patients) in medium containing the indicated amount of rIL2 prior to testing in ^{51}Cr release assays.

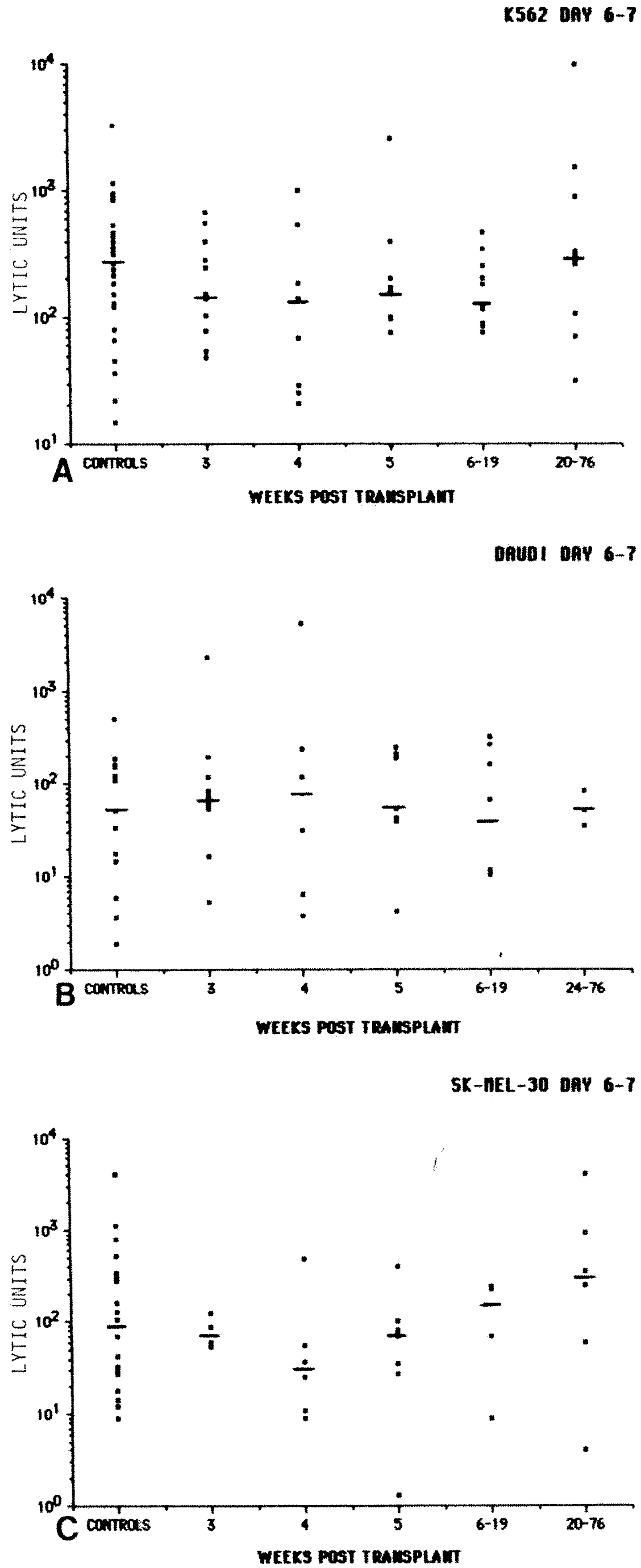

Fig 1. LAK activity of patient and control PBMCs following 6 to 7 days of culture in rIL 2. PBMCs from patients were obtained during the indicated intervals following SBA$^-$E$^-$ BMT and cultured in medium containing 10% pooled HS and 100 U/mL rIL 2 as described in the Materials and Methods section and tested as effectors to ^{51}Cr-labeled K562 (A), Daudi (B), and SK-Mel-30 (C) targets. Results are expressed as lytic units/10^8 cells; horizontal line represents the median responses. There were no significant differences between the patients or controls as measured by the Mann-Whitney U test.

Table 3. LAK Activity Following SBA^-E^- BMT

	Day 6-7*			Day 3-4*	
Posttransplant	K562	Daudi	SK-Mel-30	K562	SK-Mel-30
Controls	278 (29)†	51 (13)	88 (20)	39 (25)	33 (26)
	15-3,257‡	2-496	9-4,021	0.1-102	0.1-155
Week 3	147 (12)	66 (13)	73 (4)	101 (13)§	144 (13)§
	1-669	1-2,321	53-122	2-363	8-408
Week 4	135 (8)	74 (6)	31 (6)	245 (8)§	141 (8)§
	21-1,014	4-5,210	9-491	3-9,196	23-1,081
Week 5	154 (11)	53 (7)	74 (4)	187 (7)§	230 (7)§
	10-2,500	4-247	1-403	59-794	35-304
Weeks 6-20	154 (12)	40 (8)	99 (6)	359 (10)§	152 (14)§
	75-468	10-263	9-245	5-10,122	10-3,678
Weeks 21-76	309 (8)	52 (4)	200 (5)	208 (5)§	100 (5)§
	31-9,687	35-85	59-4,015	54-1,309	10-3,568

PBMCs from patients and controls were cultured for the indicated time in 100 U/mL rIL 2. Results are expressed as lytic units/10^6 cells.

*Number of days in culture.

†Median lytic units, number of patients tested is shown in parentheses.

‡100% range.

§$P < .05$, Mann-Whitney U test.

activated effector cells. As shown in Table 5, cold K562 and cold SK-Mel-30 (C:H ratios of 20:1) were nearly equally able to compete for hot SK-Mel-30 and hot K562, respectively, in all experiments performed. PHA blasts from a normal control, however, were unable to compete for the killing of labeled K562 or SK-Mel-30 in either of two patients tested. Therefore, cells responding to IL 2 during the early posttransplant period apparently have not been primed to either donor or host cells, and the activated killer population apparently binds equally well to the NK-sensitive and the NK-insensitive cultured tumor targets.

Effect of TBI on NK and LAK. During the course of total body irradiation (TBI) given in eight fractions over a period of 3 days, we obtained blood specimens from three patients before, at the midpoint (720 rad) and immediately after the completion of 1,440 rad TBI to determine the in vivo effect of irradiation on NK activity and on LAK precursors (Table 6). We tested the NK activity of the freshly isolated PBLs and placed cells in culture with rIL 2 for 72 hours before performing LAK assays. The NK activity of PBL derived from two patients progressively increased during TBI and was maintained at a high level in the third patient (722). LAK activity, however, could not be generated from PBLs from patient 607 following 720 rad TBI or in patients 627 and 722 following 1,440 rad. Prior to transplantation, the patients were further cytoreduced with cyclophosphamide but due to the extremely low peripheral counts following this treatment further pretransplant NK and LAK studies could not be performed. At day 18 post BMT, LAK activity could be generated following culture in rIL 2 in PBLs from patient 627 and, when tested at day 30, NK activity was completely normal. Cytogenetic analysis of bone marrow from this patient at day 14 posttransplant revealed 1 host and 3 donor metaphases; by day 30 only 27 donor and only 2 host metaphases were observed.

In addition to NK and LAK studies, PBL from patient 722 were tested by one-color and two-color immunofluorescence to determine the phenotype of the surviving cells. The results

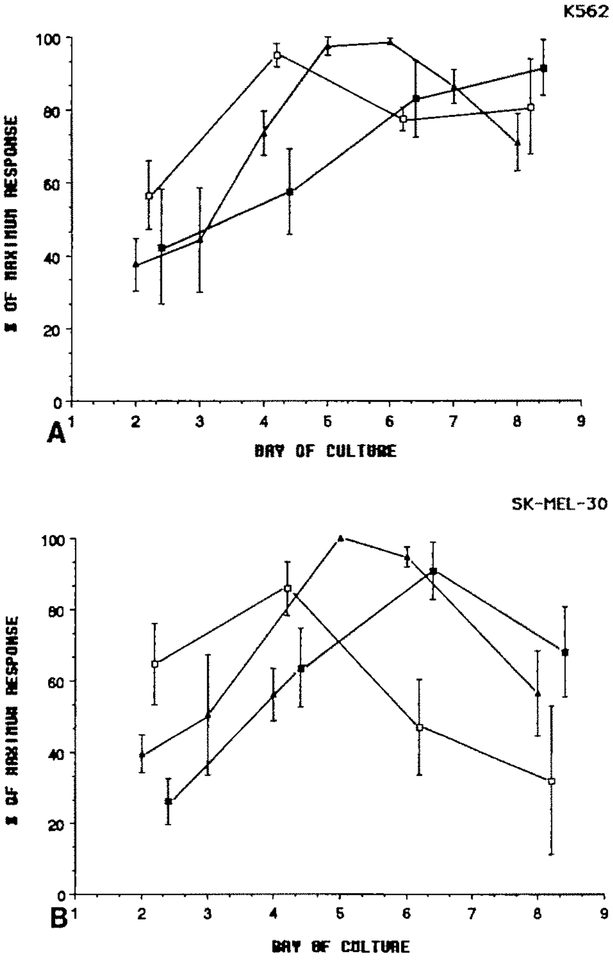

Fig 2. Kinetic response to rIL 2 induction of LAK activity of PBMCs from normal controls (—▲—) (n = 12); from patients <5 months after SBA^-E^- BMT (—□—) (n = 9); and patients >6 months posttransplantation (—■—) (n = 6). Results are expressed as the percentage ± SEM of the maximum cytotolytic response to K562 (A) and SK-Mel-30 (B) when assayed on the day indicated.

Table 4. Lysis of Peripheral Blood Targets by LAK Cells From Patients Following SBA$^-$E$^-$ BMT

			Specific Lysis (%)* Targets†			
UPN	Disease	Week Posttransplant	Host PHA	Donor PHA	K562	Daudi
473	CML	8	0.3 ± 0.2	0.4 ± 0.2	71.0 ± 3.4	50.0 ± 4.3
484	AML	5	6.0 ± 0.2	0.5 ± 0.3	88.0 ± 5.5	91.0 ± 2.3
483	ALL	5	0.3 ± 0.5	3.2 ± 1.6	67.0 ± 4.1	43.2 ± 2.8
485	MYD	4	5.4 ± 1.7	NT	83.0 ± 5.2	37.2 ± 2.4
716	AML-1	9	5.9 ± 1.7	2.6 ± 0.4	64.8 ± 6.2	55.6 ± 6.1
Control CTL†			52.5 ± 6.7	29.7 ± 3.6	42.8 ± 6.9	NT

PBMCs from patients were cultured for 6 days prior to LAK assay.

*Effector/target cell ratio 50:1; results are expressed as percentage of specific lysis ± SD of triplicate cultures.

†Patient PBMCs were frozen prior to transplant, and donor PBMCs were frozen on the day of transplant; both were thawed and cultured for 3 days in the presence of PHA before use as targets in ^{51}Cr release assays.

‡Control CTLs were generated in the presence of rIL 2 by 6-day coculture with irradiated donor PBMCs from patient 716.

Table 5. Cold-Target Competition for LAK Effectors

				Specific Lysis (%) (% of Inhibition)* Cold Targets†			
UPN	Disease	Week Posttransplant	Hot Targets	None‡	K562	SK-Mel-30	PHA§
Control	—	—	K562	26.6	4.2 (84.2)	3.9 (85.3)	—
			SK-Mel-30	25.7	5.2 (80.0)	5.0 (80.5)	—
Control	—	—	K562	51.7	12.1 (76.6)	11.3 (78.1)	—
			SK-Mel-30	41.3	16.7 (59.6)	21.2 (51.3)	—
515	ALL	4	K562	50.0	22.8 (54.4)	29.8 (40.4)	50.2 (0.0)
			SK-Mel-30	26.2	9.0 (65.6)	7.0 (73.3)	27.9 (0.0)
517	CML	3	K562	80.2	55.0 (31.4)	63.2 (21.2)	74.1 (7.6)
			SK-Mel-30	54.2	35.6 (34.3)	25.0 (53.9)	50.0 (7.7)
536	CML	4	K562	83.6	57.5 (31.2)	52.2 (37.6)	—
			SK-Mel-30	72.0	36.0 (50.0)	36.2 (49.7)	—

PBMCs from patients and controls were cultured for 6 to 7 days in medium containing rIL 2 prior to assay as described in the Materials and Methods section. Effector/target cells ratio in all ^{51}Cr release assays was 10:1.

*Percentage of reduction in lysis in the presence of cold targets.

†Unlabeled (cold) targets were mixed with ^{51}Cr-labeled (hot) targets at a ratio of 20:1 prior to addition to plates containing LAK effectors.

‡Percentage of specific lysis in the absence of cold targets.

§Unlabeled 3-day PHA blasts prepared from normal control PBMCs.

Table 6. Effect of In Vivo Irradiation on NK and LAK Activities

			Specific Lysis (%)*		
			Assay: NK (100:1)†	LAK (10:1)†	
UPN	Day‡	Rad	Targets: K562	K562	SK-Mel-30
607	−10	0	11.9 ± 2.1	32.7 ± 1.9	40.3 ± 3.4
	−8	720	18.8 ± 1.9	1.1 ± 0.3	3.2 ± 0.7
	−6	1,440	37.2 ± 3.1	—	—
722	−8	0	71.2 ± 9.0	77.8 ± 4.4	60.4 ± 1.7
	−7	720	50.1 ± 2.6	—	—
	−5	1,440	35.5 ± 2.9	1.6 ± 0.2	−2.2 ± 0.1
627	−9	0	38.6 ± 4.0	63.7 ± 2.8	57.2 ± 5.4
	−7	720	40.9 ± 2.9	13.2 ± 1.6	33.7 ± 3.2
	−5	1,440	59.4 ± 5.1	−4.0 ± 0.6	−1.1 ± 0.1
	+18	0	—	53.3 ± 4.0	55.4 ± 4.8
	+30	0	51.4 ± 3.9	76.3 ± 4.8	65.3 ± 4.0

*Results are expressed as the percentage of specific lysis ± SD of triplicate ^{51}Cr release assays. NK assays were incubated for 12 hours; LAK assays were incubated for 4 hours.

†Effector/target cell ratio.

‡Number of days before or following BMT.

shown in Table 7 indicate that the majority of PBLs were T3$^+$, T11$^+$ T cells, with 20% and 6% NKH1$_A$$^+$ cells surviving 720 and 1,440 rad, respectively. The proportion of Leu11$^+$ cells increased somewhat following 720 rad, with more of the cells coexpressing NKH1$_A$ (69% following 720 rad *v* 40% before irradiation) but were undetectable following 1,440 rad. None of the NKH1$_A$$^+$ cells jointly expressed T3 at either irradiation dose.

Presence of LAK precursors in bone marrow fractions. To determine if the rapid reappearance of LAK cells following SBA$^-$E$^-$ BMT was due to mature precursors transfused with the graft itself, we tested fractions of bone marrow from four donors in LAK assays (Table 8). There was little LAK activity following 4 days of culture in IL 2 of unseparated marrow from one donor, but a selective enrichment of LAK precursors was observed in the low-density, E rosette-positive fraction obtained following SBA treatment (SBA$^-$E$^+$) of this donor as well as in SBA$^-$E$^+$ fractions from two additional donors tested. The SBA$^-$E$^-$ fractions (the bone marrow graft), in contrast, were severely depleted of cells that could be activated to lyse the LAK target SK-Mel-30. The IL 2-activated SBA$^-$E$^-$ fractions were more variable in their ability to kill the NK-sensitive target K562 with specific lysis at an E:T ratio of 25:1 ranging from 8.6% following 6 days of culture to 29.6% on day 5 of culture. This variability may reflect quantitative differences in the depletion of mature LAK precursors or may be due to the generation of NK cells from immature precursors in the SBA$^-$E$^-$ fractions.[22,23] Maximum K562 lysis by SBA$^-$E$^-$ fractions was much less, however, than that by LAK cells from patients at week 3 post BMT following 3 days (E:T 10:1 = 46.7 ± 4.5) or 6 days (51.1% ± 6.3%) of culture in rIL 2.

Effect of Tac MoAb on LAK generation. IL 2-mediated proliferative responses can be blocked by the addition of MoAb directed to the α chain of the IL 2 receptor, Tac.[24] Conflicting results have been reported, however, concerning the ability of Tac MoAbs to inhibit the IL 2-mediated activation of NK cells[25-27] or LAK cells.[28,29] Table 9 shows the results following 3-day cultures of PBLs from two patients <8 weeks post SBA$^-$E$^-$ BMT and from one control in the presence of the indicated concentrations of Tac plus rIL 2. The lytic activity of control PBLs as well as patient PBLs could be inhibited to varying degrees by Tac but could not be completely blocked. PBLs from the patient with the lowest lytic activity were most effectively blocked by Tac (93.6% inhibition).

Table 7. Phenotype of PBLs During TBI

	Positive Cells (%) Single-Color Analysis*						Positive Cells (%) Two-Color Analysis of			
	CD3	CD2		CD16	CD20		T11$^+$	NKH1$_A^+$		B1$^+$†
Rads	T3	T11	NKH1$_A$	L11	B1	DR	T3	T3	L11	DR
0	70	92	20	14	2	15	76	0	40	100
720	64	92	20	26	2	10	70	0	69	100
1,440	91	100	6	0	0	6	90	0	0	0

BMCs were isolated from patient 722 before, during, and immediately following TBI.

*Values for single-color fluorescence represent percentage of the total lymphoid population positively reacting with the test antibody minus the percentage of cells exhibiting staining with an irrelevant control antibody.

†Values for two-color fluorescence represent percentage of indicated subpopulation of cells expressing the second cell surface antigen.

Phenotype of the LAK precursors and effectors. PBLs from both patients and controls were treated with MoAbs plus rabbit complement prior to culture in rIL 2 to identify the phenotype of the LAK precursor or following culture to identify the LAK effector. Table 10 shows the results from 11 experiments. In no experiment were we able to decrease the generation of LAK cells significantly with depletion of OKT3-, OKT4-, or OKT8-positive cells from PBLs of patients or controls. In agreement with the results of Grimm et al,[30] treatment with OKT3 generally resulted in increased LAK activity. In our studies, however, removal of OKT11$^+$ cells did not enhance activation. We were able to deplete LAK precursors to varying degrees by treatment with MoAbs directed to antigens present on NK cells, eg, OKT11,

Table 8. Generation of LAK Cells From BM Fractions

			Specific Lysis (%)*					
			K562			SK-MEL-30		
Experiment	Fraction	Day of Culture	E:T = 25:1	10:1	1:1	25:1	10:1	1:1
1	Unseparated†	4	4.8	3.1	3.0	12.4	6.6	3.0
	SBA$^-$E$^+$‡		33.3	39.1	11.3	59.1	45.7	13.2
	SBA$^-$E$^-$§		13.0	5.4	1.0	4.9	4.1	3.4
2	SBA$^-$E$^+$	4	49.4	35.2	7.1	57.2	25.7	3.7
	SBA$^-$E$^-$		18.8	7.1	4.9	15.1	7.1	2.9
3	SBA$^-$E$^+$	5	110.5	103.1	92.7	40.0	29.5	14.9
	SBA$^-$E$^-$		29.6	20.2	8.4	9.0	3.9	3.6
4	SBA$^-$E$^-$	6	8.6	2.0	0.0	1.2	0.0	0.0

*Results are expressed as the mean percentage of specific lysis of triplicate ^{51}Cr release assays.

†Bone marrow cells were removed prior to treatment with soybean agglutinin (SBA) and sheep RBCs. Low-density bone marrow cells were obtained by separation over Ficoll-Hypaque and were cultured in medium containing 100 U/mL rIL 2 and 10% pooled human serum for the indicated number of days as described in the Materials and Methods section.

‡Cells were obtained from NH$_4$CL-lysed pellets following treatment of the bone marrow with SBA and SRBC. Low-density cells were cultured as described above.

§Low-density, SBA-negative, and E rosette-depleted bone marrow cells cultured as described.

Table 9. Effect of Anti-TAC on The Generation of LAK Activity

UPN	Week Posttransplant	Target	Lytic Units* IL-2	Lytic Units* + Anti-Tac†	Ascites Dilution	Inhibition (%)
Control	—	K562	341.4	98.3	1:5,000	71.0
		SK-Mel-30	118.1	35.0	—	70.6
566	4	K562	20.4	3.0	1:250	86.0
		SK-Mel-30	11.2	0.7	—	93.6
546	5	K562	475.2	159.1	1:500	67.0
		SK-Mel-30	305.3	208.0	—	31.8

LAK cultures were initiated in the presence of the indicated dilution of anti-Tac ascites. A final dilution of 1:5,000/10^6 cells gives maximal fluorescence intensity in indirect assays.

*Results are expressed as the number of lytic units/10^8 cells.

†IL 2 plus anti-Tac.

Leu 11b, and NKH1_A, although no single treatment completely eliminated the generation of LAK cells. We were unable to reduce the activity of LAK effector cells by treatment with MoAbs to CD3 or CD8 plus complement. As in the precursor depletion experiments, the only MoAbs effective in reducing lytic activities were OKT11, Leu 11b, and NKH1_A, the most effective MoAb being NKH1_A.

Correlation of LAK activity with clinical parameters. LAK cells were generated by culture in IL 2 of PBMCs from all patients tested. There were differences in the lytic activity of individual patients and, as shown in Fig 1 and Table 3, some patients had very high activity. Because patients were tested following 6 to 7 days of culture as well as after 3 to 4 days, they were analyzed separately to determine if the level of LAK activity differed among the groups. Although some groups were small, there appeared to be no correlation of LAK activity with disease, age, speed of engraftment (day to ANC of 1,000) or the incidence of infections during the posttransplant period. Too few patients have been accrued and followed for a sufficient period to assess whether there is any correlation between quantitative aspects of LAK cell regeneration and leukemic relapse. This study is currently in progress (data not shown).

DISCUSSION

The course and kinetics of immunologic reconstitution following SBA$^-$E$^-$ BMT would be expected to differ from that following a conventional transplant, since the contribu-

Table 10. Phenotype of LAK Precursors and Effectors

UPN	Week Posttransplant	Target	Specific Lysis Complement Control (%)	MoAb + Complement (% of Complement Control)* (CD3) OKT3	(CD4) OKT4	(CD8) OKT8	(CD2) OKT11	Leu11	NKH1_A
Precursors†									
Normal		K562	16	177	165	93	—	63	36
control		SK-Mel-30	24	150	100	71	—	57	36
Normal		K562	42	138	—	—	62	30	—
control		SK-Mel-30	32	156	—	—	61	71	—
558	7	K562	68	82	90	81	68	72	34
		SK-Mel-30	63	111	84	88	51	74	33
556	6	K562	41	176	105	105	31	25	45
		SK-Mel-30	29	244	119	119	20	11	56
546	4	K562	99	—	—	—	—	58	—
		SK-Mel-30	97	—	—	—	—	82	—
Effectors‡									
Normal		K562	24	72	—	96	25	17	12
control		SK-Mel-30	32	63	—	130	39	60	50
Normal		K562	30	83	—	112	34	73	24
control		SK-Mel-30	43	83	—	86	55	77	19
562	3	K562	55	124	87	108	65	62	—
		SK-Mel-30	68	98	87	97	60	68	—
559	6	K562	16	140	—	—	39	16	—
		SK-Mel-30	24	109	—	—	41	37	—
517	3	K562	63	123	—	—	80	71	—
		SK-Mel-30	70	98	—	—	72	51	—
553	5	K562	55	—	—	—	—	80	9
		SK-Mel-30	59	—	—	—	—	85	13

*Effector/target cell ratio in all assays was 10:1. Results are expressed as percentage of complement control.

†PBLs were treated with MoAb and complement prior to culture in rIL 2 for 72 to 96 hours.

‡PBLs were incubated with MoAb and complement following culture in medium containing IL 2 as described.

tion of mature cellular elements from the graft[31] and the effects of GVHD prophylaxis[32] as well as GVHD itself,[4,5,33] are largely eliminated. Indeed, in our studies of patients post SBA$^-$E$^-$ BMT, we have demonstrated a delay in the regeneration of IL 2-sensitive T cells capable of proliferating in response to mitogen.[8] Unlike recipients of conventional marrow, however, recipients of SBA$^-$E$^-$ BM grafts transiently develop a population of T cells that spontaneously secrete IL 2 early in the posttransplant period. We were therefore interested in determining whether other cell populations, such as NK cells or LAK cells that might be sensitive to exogenous or endogenously produced IL 2, emerge during the early stages of immunologic reconstitution.

Ault et al[33] have demonstrated that the predominant population of lymphoid cells appearing between days 10 and 20 following CD5$^+$ T cell-depleted BMT, have the phenotype and function of NK cells. Similar early recovery of NK function has been reported following transplantation of both conventional marrow[35,36] and bone marrow depleted of T cells by MoAb plus complement.[37,38] Likewise, we found that as early as 3 weeks post SBA$^-$E$^-$ BMT, lysis of K562 by PBMCs from patients was equal to that by normal control NK cells. In the early posttransplant period, we also observed increased lysis of two NK-insensitive targets, the Burkitts' lymphoma cell line Daudi and an adherent melanoma cell line SK-Mel-30 by patient PBMCs as compared with concurrently tested normal controls. Similar enhanced lytic activity toward an NK-resistant B lymphoblastoid cell line was reported by Rooney et al[37] in patients 12 to 14 weeks following transplantation of bone marrow depleted of T cells by two T cell-specific MoAbs and complement.

The development of IL 2-inducible nonspecific cytotoxic cells (LAK) during the posttransplant period was examined in a murine system by Merluzzi et al,[38] who showed that LAK cells could be generated at 2 weeks postsyngeneic BMT, preceding by 2 weeks the capability to mount a specific CTL response. We found a similar early appearance of LAK precursors 3 weeks following SBA$^-$E$^-$ BMT. Cultures of patient PBMCs in rIL 2 for 6 to 7 days resulted in LAK effectors with activities equal to or only slightly lower than those from a normal control group. Following 3 to 4 days of culture in rIL 2, however, the lytic activity of patient LAK cells was significantly greater than LAK cells from the controls. We found that unlike those of controls or patients late after transplant, the LAK precursors developing during the first 5 months posttransplant had a maximal response after 3 to 4 days of culture in rIL 2. PBMCs from normal controls and patients tested between week 20 and week 76 posttransplant generated peak LAK activity after 5 to 6 days of culture, as has been reported for cancer patients and normal controls using noncultured tumor targets.[30,39,40] This accelerated response of LAK precursors to IL 2 coupled with the capacity of lymphocytes isolated from patients early post BMT to kill NK-insensitive targets spontaneously suggest the presence of a population of cells that has been activated in vivo. This activation may be a response to the IL 2 that we have reported to be produced spontaneously by T cells following SBA$^-$E$^-$ BMT.

We questioned whether rIL 2 might activate cells within the PBLs that have autoreactivity[20] or cells with reactivity to minor alloantigens expressed on host cells.[21] To address this possibility, we tested PHA blasts prepared from donor and/or host PBMCs as targets in LAK assays. Although both autologous PBL targets as well as PHA blasts can be lysed at low levels by LAK cells from some individuals,[39,41,42] IL 2-activated effectors from our patients showed no cytolysis of either donor or host targets. Furthermore, we demonstrated in cold-target competition experiments a population of LAK cells that recognized equally well both NK-sensitive and NK-insensitive unrelated targets but not third-party PHA blasts.

The origin of the LAK precursors developing early posttransplant has not been definitively determined. Our studies of the radiosensitivity of LAK precursors in vivo suggest that this activity is more sensitive than the lytic ability of freshly isolated NK cells to the irradiation dosage (1,440 rad) used for pretransplant cytoreduction. Although we have not as yet characterized the emerging LAK cells in clonal assays as donor or host, we have observed LAK precursors as early as 18 days posttransplant from a patient who by cytogenetic analysis of bone marrow had dividing cells predominantly (94%) of donor origin. The rapid regeneration of LAK activity posttransplantation does not appear to be due to enrichment of LAK precursors in the SBA$^-$E$^-$ BM graft. Our data indicate rather that LAK precursors are concentrated in the E$^+$ fraction collected following treatment with SBA. Following culture of SBA$^-$ E$^-$ marrow in medium containing IL 2, there was no substantial lysis of NK-resistant targets, although there was some lytic activity towards K562. This activity may reflect the IL 2-induced generation of NK cells from early marrow precursors as reported in murine systems.[22,23] Thus, mature LAK precursors in the host appear to be depleted by pretransplant conditioning and the early appearance of the LAK response postgrafting apparently is due to the rapid growth and differentiation of donor-derived LAK precursors from more primitive cells in the SBA$^-$E$^-$ BM inoculum. Formal cytogenetic studies of LAK cells are needed, however, to rule out the possibility that primitive progenitors of LAK cells persist through cytoreduction and contribute to the LAK activities detected early post-BMT.

Recent evidence suggests that both LAK precursors and effectors derive from cell populations with the morphology and phenotype of NK cells,[40-45] although CD3$^+$ cells bearing the NKH1$_A$ antigen may have minor activity.[45] Studies by Itoh et al[43] using both positive and negative selection of Leu 11$^+$ PBLs suggested that this population of LGLs are the LAK precursors. More recently, cells within the Leu 11$^+$, NKH1$_A{}^+$ cell population have been shown to be most strongly activated by IL 2 to lyse fresh or cultured NK-resistant tumor targets and to maintain this phenotype following culture in IL 2.[44,45] Our results in both controls and patients post-BMT confirm that the LAK precursors do not bear the classic markers of mature T cells, eg, CD3, CD4, or CD8. We were able to deplete LAK precursors partially with OKT11, Leu 11b, and NKH1$_A$ plus complement, all of which are antigens present on LGLs with NK activity. Likewise in our experiments, removal of OKT3$^+$ or OKT8$^+$ effector cells did not reduce killing of either NK-sensitive or NK-resistant targets. As in our precursor experiments, however, the removal of cells with NK phenotypes did decrease effector function. The most complete depletion of activity followed treatment with the MoAb $NKH1_A$ plus complement.

We further characterized the LAK phenomenon post-BMT by examining whether IL 2 stimulated the generation of LAK cells directly by binding to its receptor (Tac)[13] on LAK precursors. During incubation with rIL 2, we previously showed a marked increase in the number of Tac$^+$ cells in cultures of PBMCs from patients and controls,[8] suggesting either induction by IL 2 of its receptor[46] or, alternatively, expansion of preexisting Tac-positive cells. Blocking of IL 2 receptors by the addition of Tac at the initiation of LAK cultures resulted in up to a 93.6% reduction in the subsequently tested lytic activity. Other investigators have reported that the enhanced lytic activity of NK cells exposed to IL 2 in short-term cultures is not blocked by Tac[25,27] and that this activation likely occurs through the newly described β chain receptor.[47] The subsequent generation of LAK activity is inhibited by Tac, however.[28,29] Therefore, the activity detected in our patients early post-SBA^-E^- BMT is more characteristic of LAK cells than of preactivated NK cells.

From our studies, both NK cells and IL 2-inducible precursors of cells which lyse both NK-sensitive and NK-resistant tumor targets but not peripheral blood targets of donor or host origin appear to emerge early after SBA^-E^- BMT. In patients, as well as in the controls we have examined, the LAK effectors and their precursors share phenotypic characteristics with NK cells and do not bear antigens present on mature cytotoxic or helper T cells. Our studies also suggest, however, that these LAK cells differ from NK cells in that they are radiosensitive and are generated by the direct action of IL 2 through the TAC surface receptor. The redeveloping populations of NK and LAK cells may contribute to the process of immunologic recovery after transplantation by providing helper factors for B lymphocytes[48,49] or by serving as antiviral effectors.[50] Their potential role in engraftment or against the reemergence of host leukemic cells[51] remains to be determined.

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Regulation of Erythropoieten Production in a Human Hepatoblastoma Cell Line

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Production of immuno and biologically active erythropoietin was documented to occur in the human hepatoblastoma cell line HepG-2. The expression of the erythropoietin gene was further verified by Northern blot analysis using a single stranded RNA probe. In vitro studies showed that erythropoietin production by these cells was not stimulated by hypoxia or cobalt chloride, but was related to the proliferative activity of the cells in culture. In addition it was found that the secretion of erythropoietin was almost completely abrogated by tunicamycin, an inhibitor of N-linked glycosylation. This effect of tunicamycin was also observed in a permanently transfected cell line that secretes erythropoietin in large quantities.

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ERYTHROPOIETIN is a hematopoietic growth factor that plays an essential role in the differentiation of committed erythroid progenitor cells into mature erythroid precursor cells.[1,2] It is a heavily glycosylated protein with a mol wt of ~34,000 Daltons, approximately one half of which is made up by carbohydrates.[3] The peptide backbone consists of 166 amino acids and contains three potential N-glycosylation sites.[4,5] In adults erythropoietin is synthetized primarily by the kidney,[6] and to a small extent by the liver.[7] In both organs the rate of erythropoietin synthesis is augmented by anemic, hypoxic and histiotoxic hypoxia.[8,9]

Although the liver is the main source of extrarenal erythropoietin in the adult organism, erythropoietin production has not been observed in physiologic short term cultures of adult hepatic parenchymal cells. Such synthesis has been claimed for cultures of adult rat Kupffer cells[10] and fetal mouse hepatocytes.[11] The amount of erythropoietin produced, however, is exceedingly small and usually not measurable unless the culture medium is concentrated up to 60-fold. Nevertheless in one study the rate of production of erythropoietin by fetal mouse hepatocytes was found to respond to hypoxia but not to cobalt chloride.[12]

After screening a number of established human hepatoma cell lines for erythropoietin production we found that the HepG-2 cell line,[13] which has served as a valuable tool for the investigation of the synthesis of a number of plasma proteins, also secretes erythropoietin. A similar secretion of erythropoietin by human hepatoma cells has been described by Okabe et al,[14] but in the present studies we were able to characterize the production of erythropoietin by HepG-2 cells by studying the effects of hypoxia, cobalt chloride, and cellular growth rate. Furthermore, using this cell line and a cell line permanently transfected with the human erythropoietin gene, we have examined the role of the carbohydrate component of the erythropoietin molecule on erythropoietin synthesis and secretion.

MATERIALS AND METHODS

Cell cultures. The human hepatoblastoma cell line HepG-2 (passages 100-160), generously provided to us by B. Knowles (Wistar Institute, Philadelphia), was routinely maintained in culture flasks (T-75 Corning) containing MEM 10% fetal calf serum (GIBCO, Grand Island, NY). For the experimental protocols the cells were transferred to 35-mm plates and grown to confluence. Each plate contained 2 mL of culture media and culture periods were routinely 48 hours in a 5% CO_2, 37°C incubator (Forma Scientific). The cell density at the time of confluency was between 1.6 and 1.8 × 10^6 cells per plate. After the termination of the incubation period, supernatants were harvested, centrifuged, and stored at −20°C until assayed. Lysates of cells were prepared by washing monolayers with 0.9% NaCl followed by the addition of proteolysis inhibitor buffer (0.05 mol/L Na citrate, 2,000 U Trasylol, 1 mol/L EDTA, 0.1 mol/L phenylmethylsulphonylfluoride [PMSF], 10 mmol/L benzamidine). The plates were frozen at −20°, thawed and homogenized with 1 mL homogenization buffer (0.1M PO_4 pH 7.6, 4.5% bovine serum albumin, 50 μg/mL leupeptin, and 1 mmol/L PMSF). The lysate was spun at 3,000 rpm for 30 minutes and the supernatant saved for assays. The studies relating to the effect of hypoxia on the HepG-2 cells were performed in a sealed modular incubator chamber (Billup-Rothenberg, CA) that was equilibrated with mixtures of CO_2, nitrogen and oxygen to give the desired oxygen tension. Cobalt chloride (Fisher) was dissolved in sterile saline and added in the desired concentration. Tunicamycin (Sigma, St Louis) was dissolved in 0.01 mol/L NaOH to a stock solution of 0.5 mg/mL. The cells were exposed for 18 hours to tunicamycin before the initiation of the experiment and tunicamycin was present in the media during the whole incubation period.

Measurement of erythropoietin. Erythropoietin levels in supernatants and cell lysates were measured by a sensitive double antibody radioimmunoassay as previously described.[8,15] Pure radiolabeled recombinant erythropoietin (Genetics Institute, Boston) was used as a tracer in the competitive assay and the antibody was developed in rabbits against human urinary erythropoietin. The lower level of sensitivity of this assay is ~2 to 4 mU/mL of sample.

Biologic erythropoietic activity of HepG-2 supernatants was determined in the hypertransfused polycythemic mice assay as previously described.[16] The level of sensitivity of this assay is ~50 mU/mL and some supernatants were concentrated five to ten times (Amicon PM-10 membranes) before being assayed.

Measurements of transferrin and albumin. Double-antibody enzyme-linked immunoassays of both human albumin and transferrin were developed in our laboratory by modification of standard methods.[17] Alkaline phosphatase conjugated goat anti-rabbit IgG (Cappel) was used as the second antibody in both assays. The

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primary antibody against transferrin was developed in our laboratory and the anti-human albumin antibody was obtained from Cappel. The lower limit of sensitivity in both assays was ~2.0 to 4.0 μg/mL.

Northern blots. Total mRNA was extracted from confluent monolayers of HepG-2 cells by a modification of the Chirgwin method.[18] Cells were directly homogenized in 4 mol/L guanidium-isothiocyanate buffer, centrifuged at 12,000 rpm for 30 minutes, and the supernatant was subsequently pelleted through a 5.7 mol/L CsCl cushion after overnight centrifugation at 35,000 rpm at 20°C in a Beckman ultracentrifuge. Poly A^+ RNA was selected by affinity chromatography onto an oligo dt-cellulose column (Pharmacia, Piscataway, NJ).[19] Northern blot analyses were carried out essentially as described by Lehrach et al.[20] RNA was denatured by formaldehyde-formamide and subjected to electrophoresis in 1.2% agarose gels. The gels were blotted into Nytran filters and hybridized against a ^{32}P labeled single stranded RNA probe.[21] This probe consists of a 660 base pair (bp) Sma I—*Eco*RI, mouse Epo genomic fragment representing 160 bp of intron IV and 500 bp of exon V inserted in a pSP65 plasmid and was provided to us by C. Shoemaker (Genetics Institute, Boston). This region was ~90% homology with the human gene. Hybridization was carried out at 65°C with probe (10^7 cpm/mL) in 50% formamide, 10% dextran sulfate (wt/vol), 20 mmol/L of $NaPo_4$, pH 6.5, 5 × SSC, 0.2% sodium dodecyl sulfate (SDS), 0.05% bovine serum albumin (BSA), 0.05% Ficoll, 0.05% polyvinylpyrrolidine (PVP), 200 μg/mL of denatured salmon sperm DNA, 1 mg/mL yeast tRNA and 100 μg/mL of each of poly A, poly I, and poly C. After 12 hours of hybridization the filters were washed at 65°C with decreasing concentration of salts starting at 5 × SSC, 0.1% SDS and final wash in 0.1 × SSC, 0.1% SDS. (SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate.) Washed filters were further digested with RNAse A as described by DeLeon et al.[22] Filters were exposed for 48 hours to Kodak XAR-5 film at −70°C using intensifying screens.

Radiolabeling experiments. Confluent monolayers of HepG-2 cells were incubated for 48 hours in the presence of ^{35}S methionine or ^{3}H mannose (Amersham, Amersham, IL) in order to measure total protein synthesis and glycolyslation, respectively. Tunicamycin was added at variable concentrations from a stock solution of 0.5 mg/mL in 0.01 mol/L NaOH. After overnight preincubation with tunicamycin the media was replaced with fresh media containing radiolabeled tracers (50 μCi per plate of either ^{35}S methionine or ^{3}H mannose). Supernatants were collected after 48 hours incubation and the incorporated label was separated from the free isotopes using gel filtration on a G-25 sephadex column, (Pharmacia PD-10, disposable columns), as modified from Gilman et al.[23] The columns were eluted with 0.15 mol/L NaCl, 0.05 mol/L PO_4 pH 7.4 buffer and aliquots were counted by liquid scintillation.

Immunoprecipitation studies were conducted in HepG-2 cells and Chinese Hamster Ovary (CHO) cells transfected with the human erythropoietin gene (Genetics Institute). Cells were incubated with 500 μCi/plate of ^{35}S cysteine in cysteine-free media (GIBCO) in the presence or absence of tunicamycin, as described above. Supernatants and cell lysates were immunoprecipitated with a mixture of two monospecific antibodies raised in our laboratory against synthetic oligo peptides corresponding to the 1-26 amino-terminal and 150-166 carboxy-terminal sequences of the human erythropoietin molecule. These antibodies were generated essentially as described by Sue and Sytkowski[24] except that the peptides were linked to keyhole limpet hemocyanine instead of albumin. Samples for immunoprecipitation were incubated for 24 hours with a final 1:100 dilution of antibodies followed by precipitation using Protein A Sepharose 4B (Pharmacia). Immunoprecipitates were analyzed by 12% SDS-PAGE (Laemelli) followed by autoradiography.[25]

RESULTS

Erythropoietin production by HepG-2 cells. Culture supernatants from confluent monolayers were assayed for erythropoietin activity using radioimmunoassay. Serial dilutions of culture media displaced radiolabelled pure erythropoietin in a parallel way to human urinary Epo. In confluent cultures erythropoietin production ranged from 40 to 150 mU/mL per 10^6 cells cultured for 48 hours. This production has been stable over more than 50 cell passages. The erythropoietic material was found also to be active in vivo as measured by the hypertransfused mice assay. Slightly lower values were observed with the bioassay suggesting the presence of some desialated material.

Cell growth and erythropoietin production. The influence of growth rate on erythropoietin production was studied in cultures maintained for four consecutive three-day periods. Cultures were initiated at a low cell density and high proliferative rate and maintained until overconfluent. Supernatants were harvested at the end of each period and replaced with new media. Parallel cultures were used to determine cell numbers (duplicate determinations of three replicas). As shown in Fig 1 maximal erythropoietin production occurs at densities between 5 and 10 × 10^4 cells/cm², at which time the cells are at their maximal proliferative activity. When cultures reach confluence (densities >20 × 10^4/cm²) there is a significant decrease in erythropoietin production.

Effect of hypoxia and cobalt on erythropoietin production by HepG-2 cells. Confluent cultures of HepG-2 cells were subjected to increasing degrees of hypoxia by incubating them for 48 hours with predetermined mixtures of different concentrations of oxygen and nitrogen in the presence of a constant 5% CO_2 atmosphere. As shown in Fig 2, a lowering of the oxygen tension from 20% to 2% induced a more than tenfold decrease in Epo production. Control cultures produced 80 ± 5 mU of Epo per 10^6/cells per mL of media and no differences in cell counts were observed.

Similarly, cobalt chloride, another known stimulus of erythropoietin synthesis in vivo, did not stimulate Epo production. As shown in Fig 3, there was a dose-dependent

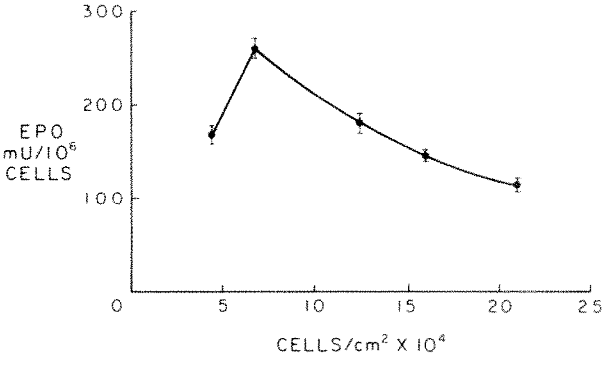


Fig 1. Dependence of Epo production on cell density. HepG-2 cells were maintained in culture for four consecutive three-day periods. The media was totally exchanged at each period interval. Epo was measured by RIA and expressed as mU/10^6 cells. Cell counts were performed in duplicate cultures.

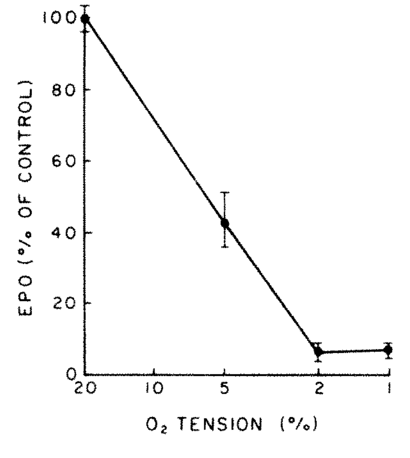


Fig 2. Effect of hypoxia on erythropoietin production. Confluent HepG-2 cells were cultured for 48 hours under conditions of variable hypoxia. Epo was measured by RIA and expressed as percent of the cultures maintained at 20% O_2.

suppression of Epo production when cells were incubated for 48 hours at increasing doses of cobalt. The levels decreased from 68 ± 5 mU/mL in the nontreated plates to nondetectable levels at the highest concentration of cobalt. The suppressive effect of hypoxia and cobalt was probably secondary to a general inhibitory effect, since similar changes were observed in the synthesis of albumin and transferrin.

Effect of tunicamycin or erythropoietin secretion. Tunicamycin, a potent inhibitor of glycosylation,[26,27] was used to study the role of the carbohydrate component of the erythropoietin molecule on its synthesis and secretion. In order to evaluate the extent of glycosylation of proteins secreted by tunicamycin-treated cultures, radiolabeling experiments were performed using ^3H mannose as a tracer. The possible inhibitory effect of tunicamycin on total protein synthesis was evaluated by measuring the incorporation of ^{35}S methionine. Separation of free and incorporated label was obtained by gel filtration. Figure 4 shows that in doses of 2.5 to 5 μg/mL tunicamycin will produce a 95% inhibition in ^3H mannose-labeled protein and a 35% inhibition of ^{35}S methionine-labeled protein.

In comparison, the specific effect of tunicamycin on the secretion of albumin, transferrin, and erythropoietin is depicted in Fig 5. While even the lowest concentration of tunicamycin (0.625 μg/mL) was associated with an almost complete abrogation of erythropoietin secretion from 51 ± 2 mU/mL to 8 ± 2 mU/mL, both albumin (a nonglycosylated protein) and transferrin (6% carbohydrates) were only moderately affected, at a rate reflecting the nonspecific inhibitory effect of tunicamycin on total protein synthesis. Intracellular levels of erythropoietin showed no significant changes in the presence or absence of tunicamycin (12 ± 2 mU/plate *v* 14 ± 2 mU/plate).

The effect of tunicamycin on the level of specific Epo mRNA was studied by Northern blot analysis of poly A+ RNA obtained from confluent HepG-2 cells cultured in the presence or absence of tunicamycin (5 μg/mL) for 48 hours. Figure 6 shows the positive hybridization signal at around 18 S obtained with a ^{32}P-labeled single-stranded RNA probe and shows that the intensity of the signal in treated and untreated cultures is equivalent. The mobility of the mRNA closely corresponds to the signal observed by Jacobs et al[5] using human fetal liver cells. However, there is also a smaller minor hybridization band that may be the result of breakdown products or represent a different species present in the tumor cell line.

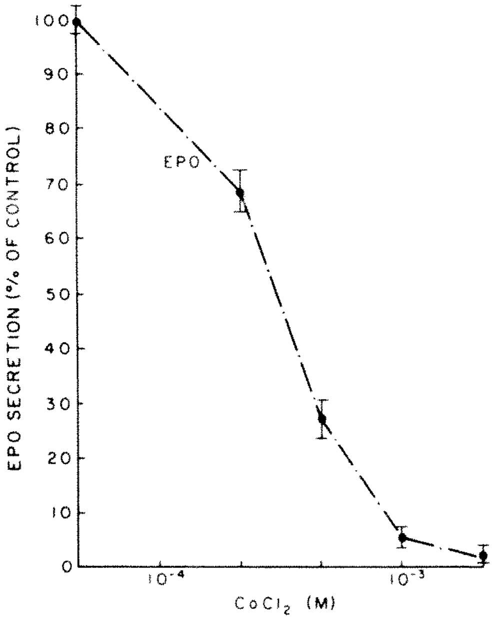


Fig 3. Effect of cobalt on erythropoietin production. Confluent HepG-2 cells were cultured for 48 hours in the presence of cobalt chloride at variable concentrations. Results are expressed as percent of cultures in the absence of added cobalt.

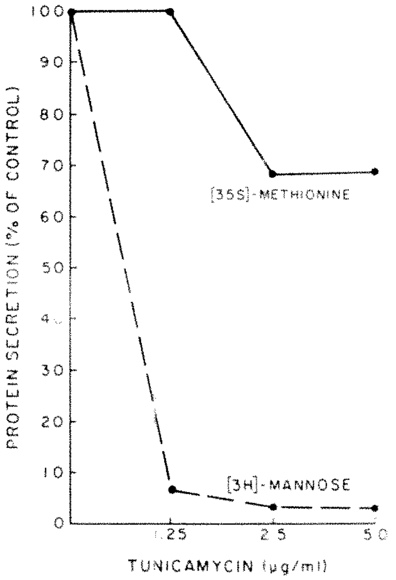


Fig 4. Dose-response curve of the effect of 48 hours exposure to tunicamycin on total protein synthesis (^{35}methionine ●——●) and glycosylation (^3H mannose ●-----●). Free and incorporated tracers were separated by gel filtration. Values are expressed as percent of secreted proteins in the absence of tunicamycin.

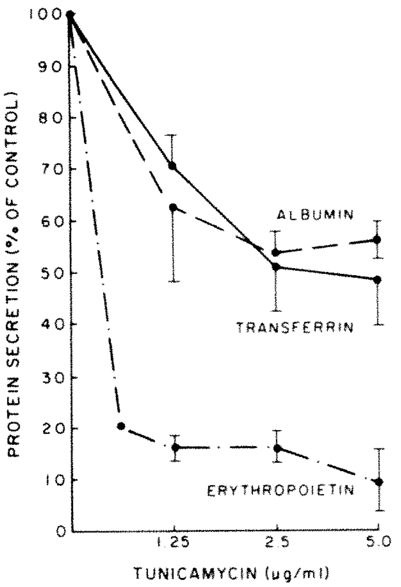


Fig 5. Effect of tunicamycin on the secretion of erythropoietin, albumin, and transferrin. HepG-2 cells were exposed to different concentrations of tunicamycin for 48 hours and supernatants were assayed for each specific protein by RIA or ELISA. Values are mean ± SD and are expressed as percent of protein secreted in the absence of tunicamycin.

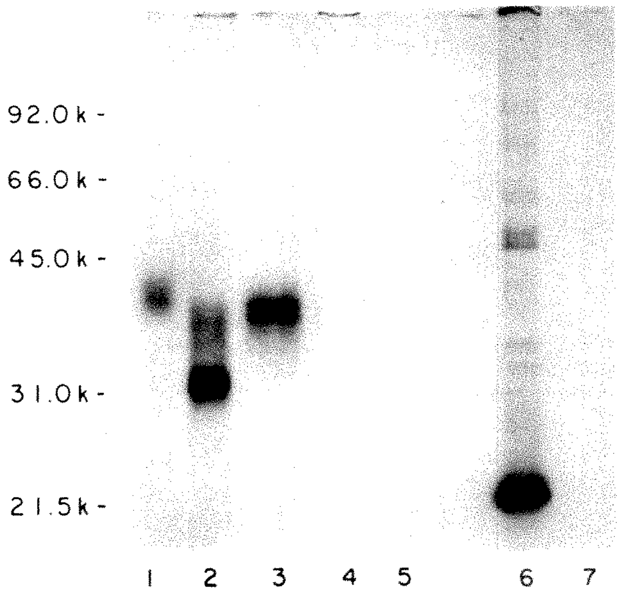
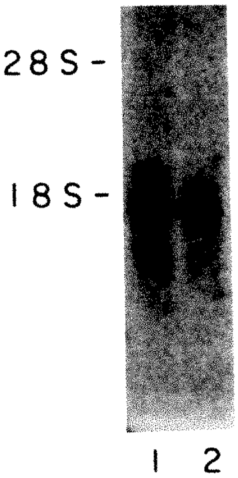


Fig 7. Immunoprecipitation of CHO cells transfected with the human Epo gene. CHO cells were incubated with ^{35}S cysteine in the presence (5 μg/mL) or absence of tunicamycin. Supernatants and cell lysates were immunoprecipitated with specific anti-Epo antibodies followed by SDS-PAGE and autoradiography. Lanes: (1) Radioiodinated pure Epo; (2) cell lysate of transfected cells; (3) supernatant of transfected cells; (4) cell lysate of nontransfected cells; (5) supernatant of nontransfected cells; (6) cell lysate of transfected cells with tunicamycin; (7) supernatant of transfected cells with tunicamycin.

To provide further evidence of the specific suppressive effect of tunicamycin on erythropoietin secretion by mammalian cells, immunoprecipitation studies were conducted in CHO cells transfected with the human erythropoietin gene, since the relatively low level of expression of erythropoietin in the HepG-2 cells precluded the experiment in this cell line. CHO cells were incubated with ^{35}S cysteine (500 μCi/plate) in the presence and absence of tunicamycin (5 μg/mL). Supernatant and cell lysates were immunoprecipitated with specific antierythropoietin antibodies obtained against erythropoietin oligopeptide sequences. Figure 7 shows no immunoprecipitable material in the tunicamycin-treated culture supernatants (lane 7) as compared with the nontreated control (lane 3). Furthermore, intracellular erythropoietin appears to accumulate in the treated cells as a major strong single band with an apparent mol wt of ~21.5 kd (lane 6) compared with an apparent mol wt of ~36.0 kd of the mature molecule (lane 2). At ~45 kd there is also a minor band that may represent aggregates of aglyco-Epo or, more likely, nonspecific coprecipitation of other intracellular labeled proteins. Interestingly, intracellular erythropoietin in the nontreated cells showed the presence of two forms, one with the apparent mol wt of the mature erythropoietin and another form with a slightly lower mol wt. This latter band probably represents erythropoietin molecules with immature glycosylation patterns. Further studies using Endo H will be necessary to clarify this point.

Fig 6. Northern blot analysis of Poly A$^+$ RNA from HepG-2 cells. RNA was extracted from HepG-2 cells cultured in the presence or absence of tunicamycin for 48 hours. After electrophoresis and transfer to nylon filters they were hybridized with a single-stranded ^{32}P mRNA probe. Lane 1 (control) and lane 2 (tunicamycin 5 μg/mL) show radioautography of 10 μg per lane of Poly$^+$ RNA.



DISCUSSION

The liver is the main site of extrarenal erythropoietin production in adult animals[28,29] and may play a predominant role in the production of erythropoietin during fetal life.[30] Increased erythropoietin production has been observed in patients with hepatomas and in accord with a previous report by Okabe et al[14] the present study showed that certain human hepatoblastoma cells can produce erythropoietin. Our cell line, HepG-2, established originally by Knowles et al[13] from a child with an hepatoblastoma, has been found to secrete a number of plasma proteins, especially high levels of alpha feto-protein. Production of erythropoietin was documented with both radioimmune assay and in vivo bioassay and by the presence of specific mRNA in northern blot analysis.

In vivo hypoxia constitutes a potent stimulus for erythropoietin production both by the kidney and the liver. Such hypoxic stimulus has also been found to induce Epo synthesis in cultures of fetal liver cells.[12] In the present study, however, cultures of HepG-2 cells failed to respond to hypoxia with increased Epo production. On the contrary there was an actual decrease in Epo synthesis as the oxygen tension was lowered. Furthermore, cobalt chloride, a pharmacologic stimulator of Epo production, also failed to elicit a stimulating response. The reason for this lack of response remains obscure but is compatible with the contention that hormone production in malignant cells is not subjected to the regulatory mechanisms operating under physiologic conditions.

Several in vivo studies have shown that hepatic erythropoietin synthesis is enhanced in conditions of accelerated cellular proliferation. This phenomenon has been observed after partial hepatectomy, hepatocyte injury, or in the recovery phase of acute hepatitis.[31-33] In our cell line it was found that peak erythropoietin production coincided with peak proliferative activity. It is tempting to suggest that the activation of the gene in the regenerating liver and in the cell line may be mediated by a similar mechanism.

The availability of this cell line has offered the opportunity to explore the biological significance of the carbohydrate component of the erythropoietin molecule on its intracellular processing and secretion. The role of *N*-linked glycosylation was examined with the use of tunicamycin, which blocks the addition of *N*-acetyl glucosamine to dolichol phosphate and thereby inhibits the formation of the core oligosaccharide.[34] In the presence of tunicamycin the secretion of erythropoietin was almost completely abrogated. In contrast, secretion of another glycoprotein, transferrin, and a nonglycosylated protein, albumin, were only moderately affected.

Measurements of specific erythropoietin mRNA in treated and nontreated cells showed no difference in hybridization signal between the two groups, suggesting that tunicamycin had no specific effect on transcription, but was acting at a posttranscriptional or secretory level.

To confirm the impression that the effect of tunicamycin was due to a lack of secretion of the nonglycosylated protein, supplementary studies were conducted in CHO cells transfected with the human erythropoietin gene. These cells secrete about a thousand-fold more Epo than HepG-2 cells and were therefore suitable for immunoprecipitation studies. The studies in CHO cells confirmed the lack of secretion of erythropoietin and demonstrated that tunicamycin-treated cells did synthesize the hormone that was accumulated intracellularly.

In conclusion we describe a human hepatoblastoma cell line that constitutively secretes erythropoietin into the culture media. Erythropoietin synthesis in this cell line appears not to be regulated by the physiologic modulators of erythropoietin production such as hypoxia or cobalt; however, it is related to the proliferative activity of the cells. Using this cell line and permanently transfected CHO cells, we have shown that secretion of the erythropoietin molecule depends on intact *N*-linked glycosylation.

ACKNOWLEDGMENT

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Recovery of Contact Hypersensitivity Responses Following Murine Bone Marrow Transplantation: Comparison of Gamma-Irradiation and Busulfan as Preparative Marrow-Ablative Agents

By Wolfram E. Samlowski and Carol L. Crump

Bone marrow transplantation (BMT) is often followed by significant morbidity and mortality due to protracted immunodeficiency. We have hypothesized that the bone marrow-ablative regimen may delay the recovery of normal immune function following transplantation by impairing the interaction of host endothelial cells with circulating graft-derived lymphocytes. This report compares the relative effects of busulfan (an alkylating drug) and gamma-irradiation on the tissue-specific localization potential of lymphocytes and the eventual recovery of immune function within syngeneic murine transplant recipients. Localization of normal lymphocytes into peripheral lymph nodes of irradiated BMT recipients was markedly less (<50%) than in busulfan-treated or normal mice over the first 2 months post-BMT. This finding correlated with irradiation-induced endothelial cell edema and microvascular occlusions within lymphocyte-receptive areas of the nodal microvasculature. The effect of both preparative regimens on the recovery of contact hypersensitivity (CHS) was also analyzed. This response recovered more quickly (between 1 and 2 months) in busulfan-pretreated animals. Further experiments demonstrated that the decrease in CHS responsiveness appeared, in part, related to a depression in the capacity of lymphocytes to localize into skin sites of antigen deposition within irradiated mice. The impairment of tissue-specific lymphocyte localization may represent a novel mechanism by which whole body irradiation can contribute to delayed immunologic reconstitution following bone marrow transplantation.

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BONE MARROW transplantation (BMT) has gained widespread acceptance in the treatment of a wide range of clinical conditions, including congenital immunodeficiency, aplastic anemia, and leukemia.[1,2] While representing a major advance in the treatment of these diseases, the procedure is also associated with significant morbidity associated with severe protracted alterations in immunologic function.[3,4,5] Depressions of both humoral and cellular immune functions occur during the first 100 days after transplantation. This pan-immunodeficiency is thought to represent a consequence of the aggressive chemotherapy or radiotherapy protocols used to ablate the recipient's immunohematologic system, before donor marrow infusion.[4]

The eventual hematologic and immunologic recovery post-BMT is closely linked to the engraftment, proliferation, maturation and differentiation of donor-derived stem cells.[4] Some recovery of peripheral blood lymphocyte subsets can usually be demonstrated within 3 to 6 months of BMT.[6-9] Despite the relatively early reappearance of phenotypic T and B cells, functional immunologic recovery may take over 2 years.[3,4,7,9,10] During this period, serum antibody production, cytotoxic T cell function, mitogen responsiveness, as well as other lymphocyte functions appear to recover. Some complex immunologic responses, such as antigen-specific helper T cell function, IgA secretion, and contact hypersensitivity responses, appear to be even more delayed in their recovery.[10-13] In one study, helper T cell function was found to be depressed as late as 4 to 6 years post-BMT.[11]

The degree of histocompatibility between the bone marrow donor and recipient does not appear to significantly influence the rate at which immunologic recovery occurs following BMT. Immunologic reconstitution occurs at a similar pace following human twin, allogeneic and autologous transplants.[14,15] The development of graft-v-host disease, however, significantly worsens the severity and duration of the various cellular and humoral immune deficits observed posttransplantation.[16,17]

It is possible that current BMT protocols have detrimental effects on the recovery of the immune system posttransplantation by producing unintended effects on host tissues. We have hypothesized that some of the post-BMT immunologic deficits are produced by toxicity of the bone marrow-ablative regimen on the endothelial cells of the recipient. Alterations in the capacity of endothelial cells to bind graft-derived lymphocytes may account for decreased tissue localization potential of immune effector cells and thereby result in depressed immunologic function.

Under normal circumstances, mature lymphocytes continually migrate throughout the body, passing from the bloodstream through the various lymphoid organs and eventually returning to the bloodstream. The ability of lymphocytes to migrate through tissues is termed recirculation.[18,19] Virtually the entire lymphocyte content of peripheral lymph nodes enters the nodal parenchyma from the bloodstream via this process.[20] Tissue-specific extravasation appears to require the selective interaction of lymphocyte surface receptors with lymph node microvascular endothelial cells.[21] The adhesion process appears to take place in histologically distinct cuboidal endothelium-lined post-capillary venules, known as high endothelial venules (HEV).[22,23] Analogous tissue-specific extravasation-mediating structures are also associated with the endothelium of other lymphoid organs, such as the

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Peyer's patches,[24] mesenteric lymph nodes,[23] peribronchial lymphatic tissue,[25] and synovium.[26]

Continual lymphocyte recirculation through peripheral lymph nodes is believed to enhance the probability that a T cell, which is committed during differentiation to a single antigen epitope, can encounter its specific antigen.[23,27] The antigen recognition process is aided by the simultaneous concentration of antigen-presenting cells (APCs) within peripheral lymph nodes via the afferent lymphatic drainage.[28] The peripheral lymph nodes also contain all of the humoral and cellular regulatory elements needed to amplify or depress immune responsiveness. The peripheral lymph nodes, therefore, serve as structures that facilitate the antigen recognition process and enhance immune surveillance of host tissues.

Our previous studies established that marrow-ablative doses of gamma-irradiation (>7.5 Gy) caused distinctive ultrastructural changes in murine and human lymph node HEV.[29] These alterations in murine HEV were associated with a depressed capacity of lymphocytes to localize into irradiated lymph nodes from the bloodstream.[29] Due to the importance of peripheral lymph nodes in the initiation and regulation of immune responses, we hypothesized that this depressed localization capacity should be associated with demonstrable changes in immune function.

In this report we compare the effects of two commonly used bone marrow-ablative agents,[2] busulfan (an alkylating drug) and gamma-irradiation, on HEV histology and lymphocyte tissue localization potential. Since the two preparative regimens differed significantly in their endothelial toxicity, it proved possible to assess their respective effects on the timecourse of immunologic reconstitution following BMT.

MATERIALS AND METHODS

Animals. Six- to ten-week-old C3H/HeN mice were obtained from the animal production facility of the National Cancer Institute (Bethesda, MD). All mice were housed at a maximum density of six animals per 18 × 28 cm cage and maintained on Wayne sterilizable Lab Blox (Wayne Pet Food, Chicago) and acidified water ad libitum. Mice were age and sex matched for each experiment. Before exposure to either preparative regimen, the experimental animals were pretreated for two days with 0.5 mg of gentamycin sulfate administered intraperitoneally (IP). Throughout the course of the experiment, recipient mice also received oral nonabsorbable antibiotics (35 mg of neomycin and 2,500 units of polymyxin B per 200 mL of drinking water). This treatment was used to reduce mortality due to infection during the experiment.

Preparation of lymphocytes. Peripheral lymph nodes (PLN) (inguinal, axillary, brachial, and cervical) were excised from donor mice and gently dissociated in RPMI 1640 medium (GIBCO, Grand Island, NY) with 5% fetal bovine serum (Sterile Systems, Logan, UT) (complete medium). The cell suspension was layered onto a glass-wool column to remove adherent as well as nonviable cells. Cell suspensions were always ≥99% viable by trypan blue exclusion.

Gamma-irradiation of mice. Recipient mice were lethally irradiated with 7.5 Gy (1 Gy = 100 rad) per whole body at 4.8 Gy/min using a Gammator (Isomedix, Parsippany, NJ) containing a cesium source. Animals were reconstituted with $\sim 10^7$ syngeneic nucleated bone marrow cells immediately following irradiation. The irradiated mice were then placed into sterile cages, in a laminar airflow environment and given sterile food and antibiotic-containing water ad lib.[29]

Busulfan treatment of mice. Busulfan (Burroughs Wellcome, Research Triangle, NC), 6.25 mg, was dissolved in 200 μL dimethylsulfoxide and 800 μL warm (37°C) phosphate-buffered saline (PBS, pH 7.4). Antibiotic pretreated mice were given daily doses of 25 mg busulfan/kg body weight IP for six sequential days. This dose has been established as the hematologic LD_{100} for mice (reference 30 and unpublished observations). Animals were reconstituted with 10^7 nucleated marrow cells 24 hours following the last dose of busulfan, a point at which all active drug has been cleared from the circulation.[31] Animals were then maintained in the same fashion as irradiated BMT-recipients.

Radiolabeling of lymphocytes with [^{51}Cr] for localization studies. Lymphocytes were radiolabeled with [^{51}Cr] (as sodium chromate, specific activity 250-500 mCi/mg Cr; Amersham, Arlington Heights, IL). Lymphocyte suspensions (10^8 viable cells/mL) were incubated with 100 μCi [^{51}Cr] for half an hour at 37°C. Radiolabeled cells were washed three times in complete medium, adjusted to 10^7 lymphocytes per milliliter in RPMI and 0.2 mL was injected intravenously (IV) into normal, busulfan-treated, or gamma-irradiated recipient mice. The administration of gamma-irradiation or busulfan was timed in such a manner that the comparative localization studies involving the infusion of [^{51}Cr]-labeled lymphocytes into the various experimental groups could all be performed from a single preparation of radiolabeled cells.

Radiolabeling of lymphocytes with [^{125}I] for localization studies. Cells synthesizing DNA following antigen sensitization were labeled with [^{125}I] (as iododeoxyuridine [^{125}IUDR], Amersham, sp act 5 Ci/mg). Lymphocytes were isolated from antigen-sensitized donor mice, and a single cell suspension prepared (5×10^7 cells/mL). A total of 2 μCi of [^{125}IUDR] was incubated per milliliter of the cell suspension for two hours (37°C) with periodic gentle agitation. This was followed by extensive washing and resuspension of the cells to 10^8/mL. Each recipient was infused IV with 4×10^7 [^{125}IUDR] labeled cells.

Isotope quantitation and data presentation. Animals were killed four hours after infusion of labeled cells. The mice were autopsied, and individual organs were removed and placed into separate vials for counting in a Beckman Gamma 8000 gamma counter (Beckman Instruments, Irvine, CA), as was the remaining carcass. Data are presented as the percentage of radioactivity recovered per individual organ, as compared with the total radioactivity recovered from the experimental animal. Background values were subtracted from each one-minute count. This method of data calculation precludes errors introduced by inoculation variation between animals, as organ distribution is independent of the number of radiolabeled cells injected over a wide dose response range.[32] Localization experiments were run using two to three animal groups because of high concordance between animals. Each experiment was repeated at least twice.

Assessment of relative marrow ablative potential of irradiation and busulfan regimens. Relative bone marrow stem cell ablation produced by gamma-irradiation (a single dose of 7.5 Gy) or busulfan (25 mg/kg/d for six days, IP) was quantified by an in vitro granulocyte-macrophage colony assay (CFU-GM). One femur from each mouse within three animal groups (which had previously been exposed to one of the preparative regimens) was isolated, and the marrow irrigated out with a syringe. Each femur contains ~4.5% of the total mouse bone marrow compartment.[33] Following gentle dissociation of each animal's marrow into a single cell suspension, erythrocytes were lysed with Tris-buffered 0.16 mol/L ammonium chloride. The remaining nucleated cells were counted by phase microscopy. The CFU-GM were assessed by plating the entire nucleated bone marrow cell population from each femur into 2 mL of 0.8% methylcellulose (Dow Chemical Co, Midland, MI), in the presence of murine post-endotoxin serum (a source of GM-colony stimulating factor [GM-CSF]) at a predetermined optimal concentration.[34] After 12 days colonies of >40 cells were counted. Normal murine bone marrow cells (10^6 cells per 60 mm plate), cultured in the presence of GM-CSF served to establish the maximum colony formation potential. Bone marrow cells plated in the absence of GM-CSF served as a negative control.

Immunoperoxidase staining of frozen sections. Frozen sections were incubated for 30 minutes with MECA-325 (generously supplied by E. Butcher, Stanford, CA). MECA-325 is a monoclonal rat-anti-mouse endothelial cell antibody (IgGl), which was produced by fusing spleen cells from rats that had been immunized with mouse (Balb/c) lymph node stroma with SP 2/0 mouse myeloma cells.[35] This monoclonal antibody binds specifically to murine HEV, and not other lymph node structures.[35] No cross reaction occurs with capillaries, arteries, and arterioles that do not have the cuboidal endothelial features associated with specific lymphocyte binding.[35] To identify MECA-325 binding, a biotinylated rabbit-anti-rat antiserum (Cappel) was used as a second stage reagent. This reagent did not bind to mouse lymph node sections in parallel control slides. Immunoperoxidase staining was then performed using a modified avidin-biotin-peroxidase complex technique.[36]

Sensitization and elicitation of contact hypersensitivity (CHS). Experimental and control mice were sensitized on the shaved abdomen with 25 μL of 0.25% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma, St Louis) in a vehicle of acetone/olive oil (4:1) on two consecutive days. Four days after the first sensitization, a challenge dose of 10 μL 0.25% DNFB was applied to the right hind footpad.[37] Footpad swelling was measured (using a micrometer) 24 and 48 hours after challenge. The increment in footpad swelling of a constant area of the challenged *v* the contralateral unchallenged footpad was expressed in units of 10^{-3} inches. The increment in swelling (2 to 3 measurements/animal) for each mouse was then averaged with the remainder of the 4-5 animal group, and the results reported as the mean ± SEM.

Adoptive transfer of CHS. Normal mice were sensitized with 25 μL of 0.25% DNFB on the shaved abdomen and 10 μL DNFB on each footpad and ear on two consecutive days (donors). Four days after the initial sensitization, popliteal, inguinal, axillary, brachial, and cervical nodes were excised and gently dissociated in complete medium. The resulting single cell suspensions were injected IV into previously transplanted mice (either irradiated or busulfan-pretreated) at 4×10^7 cells/recipient. The recipients of the cell infusion were challenged with 10 μL 0.25% DNFB on the right hind footpad immediately following cell transfer. The increment in footpad swelling was determined 24 and 48 hours later. Age-matched normal syngeneic mice that received an adoptive transfer of sensitized lymphocytes served as a positive control.

In some experiments, the antigen-sensitized lymphocytes were radiolabeled with [^{125}IUDR] before their adoptive transfer. In these experiments, the animals were challenged on one ear (instead of the footpad), and the increment in ear swelling ($\times 10^{-4}$ inches) was measured only at 24 hours. Each animal was then killed and its organs (including each ear separately) assayed to determine the distribution of the infused radiolabeled cells (as described above).

Direct injection of hapten-modified lymphoid cells and effector lymphocytes into the skin. Syngeneic spleen cells were treated with buffered ammonium chloride to lyse erythrocytes (as described above). Splenocytes were trinitrophenylated by incubation of suspensions of 10^7 cells/mL in PBS containing 2.5 mmol/L 2,4,6-trinitrobenzene sulfonic acid ($TNBSO_3$) for ten minutes at 25°C. After hapten modification, the cells were extensively washed in medium and resuspended at 10^8 cells/mL to use as targets for antigen-primed effector cells. The effector cells were generated by sensitizing mice on the belly, footpad, and ears (as described above) with a single application of 7% 2-chloro-,1,3,5-trinitrobenzene (TNCB). These mice subsequently served as donors for sensitized lymphocytes four days later. The antigen-sensitized lymphocytes were also suspended at 10^8 cells/mL, and mixed 1:1 with hapten-modified target cells. Groups of irradiated BMT recipients or normal control mice were injected with 10 μL of the combined cell suspension in the right ear. The increment in ear swelling (compared with the contralateral ear) was determined 48 hours later to minimize any residual swelling due to injection trauma ($\times 10^{-4}$ inches). Mice that received an equivalent volume of sensitized effector lymphocytes without hapten-modified splenocytes served as a negative control.

RESULTS

The initial experiment was designed to compare the relative effects of irradiation or busulfan exposure on tissue localization of normal lymphocytes within BMT recipients. These experiments were performed by infusing 2×10^6 normal [^{51}Cr]-labeled peripheral lymph node lymphocytes IV into previously transplanted mice (0.2 mL/recipient). Four hours following the infusion of radiolabeled lymphocytes, the animals were killed and autopsied. Each organ (peripheral lymph nodes, spleen, mesenteric nodes, gut, liver, kidneys, lungs and the residual carcass) was counted separately in a gamma counter to determine the primary localization of the cells. The results (Fig 1) are expressed as a percentage of the injected cells that localized within the peripheral lymph nodes (±SEM), compared with the total recovery from each animal. As early as 1 week post-BMT, a time when the function of both the hematopoietic and immunologic systems of the BMT recipient remain ablated by the preparative regimen, lymphocyte localization into the lymph nodes of previously irradiated mice was <50% of controls. No evidence of lymphocyte sequestration in any other organ could be demonstrated as a cause for the depression in cells reaching the peripheral lymph nodes (data

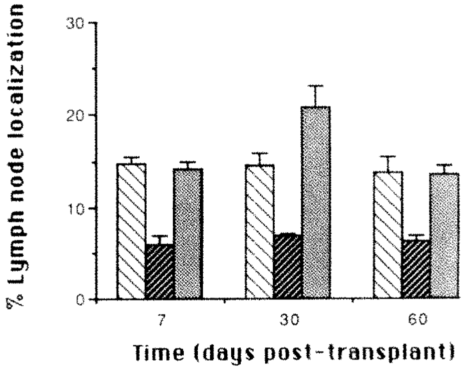

Fig 1. Localization of normal lymphocytes into the peripheral lymph nodes of BMT recipients. Mice were transplanted, using busulfan (25 mg/kg/d × 6 days) or irradiation (7.5 Gy) as the marrow-ablative regimen. Animals received 2×10^6 [^{51}Cr]-labeled normal peripheral lymph node lymphocytes as an IV infusion either 7, 30, or 60 days after BMT. Four hours after infusion of cells, the recipients were killed and autopsied. Radioactivity recovered from the pooled peripheral lymph nodes (inguinal, brachial, axillary, and cervical) is expressed as a percentage of the total radioactivity retained within each animal. The results are expressed as the average of three animal groups (±SEM). ▧, Control; ▨, irradiated; ▒, busulfan.

not shown). The decrease in lymphocyte localization into peripheral lymph nodes in irradiated mice persisted through the 1- and 2-month timepoints. These times were chosen for experimental evaluation because significant graft-derived hematologic recovery occurs by 1 month post-BMT. By 2 months following syngeneic murine transplantation, phenotypic recovery of graft-derived lymphocytes in the blood and lymphoid tissues may be observed.[38] Busulfan-treated mice did not exhibit significant depression in the capacity of lymphocytes to localize into nodes at any time tested. This suggests that busulfan, unlike gamma-irradiation, does not adversely affect the lymphocyte-receptive endothelium within peripheral lymph nodes.

These results could also be explained if the marrow-ablative dose of busulfan was less potent than that of gamma-irradiation. If this were true, less lymph node microvascular damage might also be expected. We performed an experiment to directly assess the degree of bone marrow stem cell ablation produced by both of the experimental regimens. Groups of three mice were pretreated with either busulfan or irradiation. Hematologic reconstitution by BMT was not performed. Twenty four hours after the last exposure to either preparative agent, the marrow content of one femur per animal (~4.5% of its total bone marrow)[33] was assessed for its ability to form CFU-GM. Care was taken to assess CFU-GM in busulfan-pretreated mice at a time when all drug and its active metabolites were believed to have been cleared from the circulation.[31] To establish the normal CFU-GM content per femur, a group of normal age- and sex-matched control mice served as a control. The results of this experiment (Table 1) established that busulfan-pretreatment decreased the number of CFU-GM by a factor of $>10^4$/femur, compared with 10^3 for 7.5 Gy-irradiated animals. The apparent differences in lymphocyte recirculation potential produced by the two regimens cannot, therefore, be accounted for by less intensive marrow ablation within the busulfan-treated group. These two regimens likely differ in their effects on the lymph node HEV structures.

Table 1. Cytoreductive Potential of the Irradiation and Busulfan Treatment Protocols

Group	Nucleated Cells Recovered per Femur	CFU-GM Recovered per Femur	Stem Cells Reduction by Preparative Regimen (%)
None	5.6×10^7	11,200	0
Irradiation	5.0×10^6	7.8	99.03
Busulfan	3.4×10^5	0.8	99.003

The decrement in bone marrow stem cells within animals exposed to either busulfan (25 mg/kg/d × 6 days IP) or irradiation (7.5 Gy) was quantified with the CFU-GM assay. Three animal groups were treated with each of the preparative regimens. Twenty-four hours following completion of the marrow-ablative regimen, a single cell suspension was prepared from the bone marrow contained within one femur from each animal. Erythrocytes were lysed with buffered ammonium chloride and the number of nucleated cells counted. The entire nucleated cell content of each femur (~4.5% of the total marrow mass of a mouse)[33] was then plated into 0.8% methyl cellulose in RPMI 1640 medium, 20% fetal bovine serum, and a predetermined optimal concentration of murine post-endotoxin serum (a source of GM-CSF). After 12 days, the resulting CFU-GM colonies (>40 cells) were counted. The CFU-GM content of normal femurs was established by plating 10^6 nucleated bone marrow cells, derived from age-matched controls, into culture as described above. The CFU-GM content of a normal femur was then calculated based on: (the total number of nucleated cells per femur [$\times 10^6$ cells] × (CFU-GM/10^6 cells plated into culture).

The microanatomy of the peripheral lymph node HEV was directly examined by immunochemical techniques. Frozen sections of mouse peripheral lymph nodes were prepared from animals 2 months postexposure to either busulfan or irradiation. These sections were stained with the monoclonal antibody, MECA-325, using an immunoperoxidase technique.[36] This monoclonal antibody specifically identifies lymphocyte-receptive areas of endothelium within peripheral lymph nodes (high endothelial venules).[35] The immunoperoxidase-stained sections of gamma-irradiated lymph nodes (Fig 2A) demonstrated a marked disorganization of the HEV histology, with apparent edema and proliferation of the endothelial cells, producing microvascular occlusions. Lymph nodes from busulfan-pretreated animals (Fig 2B) were found to have a normal appearing HEV architecture, with cuboidal endothelium and patent vascular lumens. A MECA-325–stained section of a lymph node from a normal animal is presented for comparison (Fig 2C). Electron microscopy (EM) was also performed at the 2-month timepoint. The EM studies confirmed our previous findings of severe intracellular edema within endothelial cells of previously irradiated HEV.[29] Lymph node endothelial cells from busulfan-pretreated mice, on the other hand, were ultrastructurally normal (data not shown).

Since the capacity of lymphocytes to enter peripheral lymph nodes appears to be impaired by prior irradiation, it follows that immunologic hyporesponsiveness might occur as a consequence. Contact hypersensitivity (CHS) was the initial response evaluated, because CHS is known to be depressed for years following BMT in humans.[3,4] CHS may be particularly susceptible to preparative-regimen–induced endothelial damage, because lymphocyte recirculation is thought to play an important role in this response. The sensitization and amplification phases of this response take place, in part, following localization of circulating lymphocytes into the region lymph nodes that drain the antigen-exposed skin.[39,40] Antigen-sensitized CHS-effector T cells that are subsequently generated in the peripheral lymph nodes are thought to exit the node via efferent lymphatics and migrate via the thoracic duct into the bloodstream. These sensitized lymphocytes exhibit the capacity to extravasate from the bloodstream specifically into sites of subsequent antigen exposure in the skin.[39,40]

The effects of the BMT-preparative regimen on the induction of CHS-responsiveness were assessed by testing groups of mice transplanted using either busulfan or irradiation 1 and 2 months previously. Mice were sensitized with DNFB on their shaved abdominal skin. Four days after the initial sensitization, the challenge dose of DNFB was applied to the right hind-footpad. The degree of swelling was measured with a micrometer 24 hours later, and compared with the contralateral normal footpad. A group of sensitized and challenged age-matched normal mice served to establish the normal range of CHS-responsiveness for each experimental timepoint. A nonsensitized group that was exposed only to

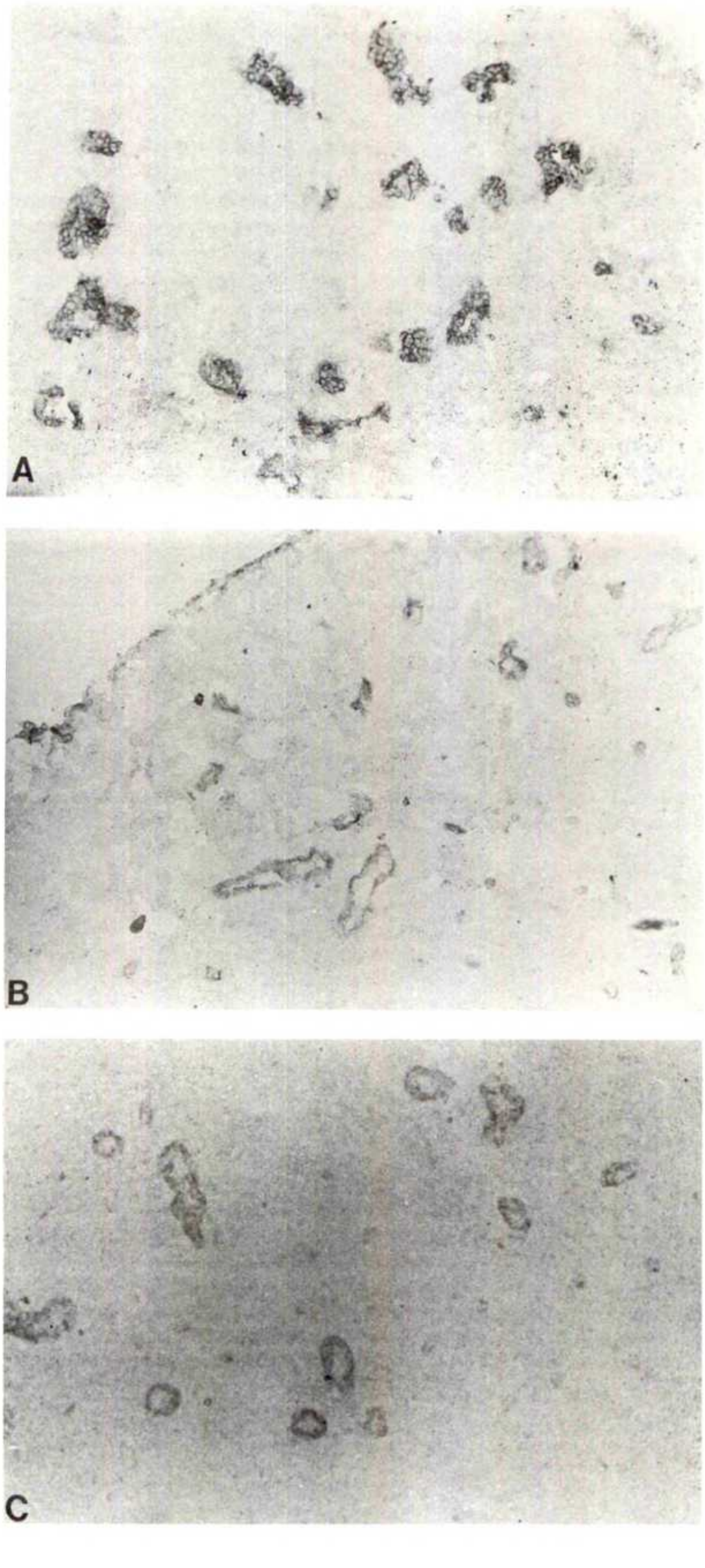

Fig 2. Immunoperoxidase staining of peripheral lymph node HEV with the monoclonal antibody MECA-325. Mice were exposed to either irradiation (A) or busulfan (B) marrow ablation, followed by syngeneic hematopoietic reconstitution. Frozen sections (6 μm) were prepared from their lymph nodes, obtained 2 months after BMT, and stained with MECA-325 by immunoperoxidase technique as described in the Materials and Methods section. The irradiated lymph nodes exhibit apparent endothelial cell proliferation, edema, and occlusion of vascular lumens, unlike busulfan exposed nodes. For comparison, a normal peripheral lymph node is also shown (C).

the challenge dose of DNFB served as a negative control. This experiment (Fig 3) demonstrated that the de novo induction of CHS-responsiveness was markedly diminished in both the irradiated and busulfan-treated groups at 1 month posttransplantation. By 2 months, however, the busulfan group had recovered a normal degree of CHS-responsiveness. The 2-month irradiated group, on the other hand, still exhibited a markedly depressed response.

These results indicate that this antigen-specific immunologic response is depressed longer in previously irradiated mice than in busulfan-pretreated BMT recipients. The elicitation of CHS responses, however, requires a complex system that involves multistep antigen sensitization, amplification, and effector phases. Any experimental result must therefore be intrepreted with caution, since irradiation could be acting at one or multiple steps in the response. Additional experiments were performed, analyzing two specific components of the CHS response, to better define some of the potential mechanisms resulting in the observed depression of CHS responsiveness.

The effector phase of the CHS response (ie, the ability of sensitized effector lymphocytes to localize into the skin from the bloodstream and produce edema) can be tested by means of an adoptive transfer experiment. Such an experiment was performed by allowing antigen sensitization to proceed in normal animals. A single-cell suspension of lymphocytes was prepared from the lymph nodes of these donor mice, and 4 × 10^7 cells/mouse were infused IV into groups of experimental animals. This experiment was performed in groups of mice transplanted 1 and 2 months previously, using either the busulfan or irradiation protocol. Mice were challenged with antigen on one footpad immediately following the adoptive transfer of cells. The increment in foodpad swelling was determined 24 hours later (Fig 4). Parallel groups of normal age-matched control mice were used at each time point to establish the maximum expected degree of swelling elicited by such an adoptive transfer of effector cells. The results of this experiment (Fig 4) demonstrate a marked reduction in the capacity of normal DNFB-sensitized effector lymphocytes to elicit swelling in footpad challenge sites in previously irradiated mice (28% and 34% of control at 1 and 2 months post-BMT, respectively). This experiment suggests that prior radiotherapy interferes with either the ability of effector T cells to localize into the skin of irradiated BMT recipients or the subsequent ability of these cells to elicit swelling within cutaneous tissues. The response was somewhat depressed at 1 month following busulfan exposure. By 2 months, however, the ability to adoptively transfer CHS responsiveness into busulfan-treated recipients was not different from normal. Measurement of footpad swelling at 48 hours (to exclude the possibility that irradiated animals exhibited a delayed, but otherwise normal, CHS response) demonstrated a similar pattern to the 24-hour measurements, but were uniformly reduced in magnitude within each group of mice.

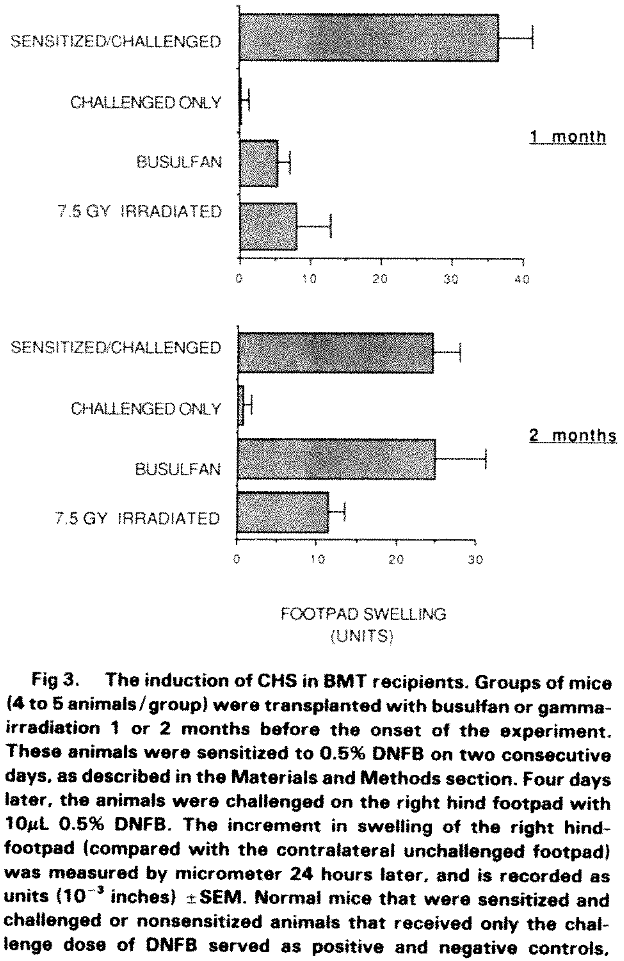

Fig 3. The induction of CHS in BMT recipients. Groups of mice (4 to 5 animals/group) were transplanted with busulfan or gamma-irradiation 1 or 2 months before the onset of the experiment. These animals were sensitized to 0.5% DNFB on two consecutive days, as described in the Materials and Methods section. Four days later, the animals were challenged on the right hind footpad with 10μL 0.5% DNFB. The increment in swelling of the right hind-footpad (compared with the contralateral unchallenged footpad) was measured by micrometer 24 hours later, and is recorded as units (10^{-3} inches) ±SEM. Normal mice that were sensitized and challenged or nonsensitized animals that received only the challenge dose of DNFB served as positive and negative controls, respectively.

The possibility that previous irradiation of the skin is responsible for the failure to produce edema was then addressed. Four days after sensitization of normal donor

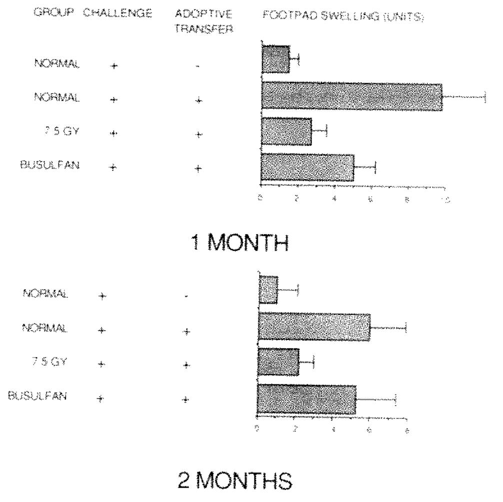

Fig 4. The adoptive transfer of CHS to previously transplanted mice. Normal mice were sensitized to DNFB and served as donors for the adoptive transfer of antigen-sensitized peripheral lymph node lymphocytes. A single cell suspension of peripheral lymph node lymphocytes from these mice (4 × 10^7 cells per recipient) was injected IV into groups of previously transplanted mice (1 or 2 months post-BMT). These recipients were immediately challenged on the right hind-footpad with 10 μL 0.5% DNFB. Twenty-four hours following challenge, the increment in footpad swelling was measured (10^{-3} inches ±SEM).

mice with trinitrochlorobenzene (TNCB), donor animals were killed and their lymph nodes excised. A single cell suspension was prepared from the sensitized lymphocytes contained within these nodes. An equal number of these cells and antigen-haptenized spleen cell targets were mixed in a small volume of media, and 10 μL injected into the right ear pinnae of mice that had been irradiated 2 or 8 months previously. The increment in ear swelling was measured with a micrometer 48 hours after injection, to minimize any degree of edema attributable to injection trauma. A parallel group of normal mice injected with sensitized effector lymphocytes and antigen served as a positive control. Animals that received only the same number of antigen-primed lymphocytes without antigen (in equivalent volume of medium) served as a negative control. The results of this experiment (Fig 5) demonstrated that the degree of ear swelling that could be elicited in both the irradiated and unirradiated groups was similar. The response was also shown to be antigen dependent, since animals that received only sensitized lymphocytes, but no antigen, did not develop ear swelling. The conclusion of these experiments is that irradiated skin is capable of responding to a local CHS stimulus with the same degree of edema as normal skin.

These experiments suggested that irradiation-induced depressions in CHS responsiveness were, in part, due to a decrease in the ability of effector lymphocytes to localize into the skin from the bloodstream. This possibility was experimentally tested by preparing a single cell suspension of DNFB-sensitized lymphocytes derived from normal mice. Proliferating cells within this suspension (presumably undergoing clonal expansion in response to antigen exposure) were labeled with ^{125}IUDR for two hours. Cells from this suspension (4 x 10^7 cells/recipient) were then infused IV into previously transplanted mice. These animals were immediately challenged with DNFB on the right ear. As in preceding adoptive transfer experiments, the increment in swelling was measured at 24 hours. The mice were subsequently killed and autopsied. Each ear (as well as other organs and carcass) were counted separately in a gamma counter to determine the percentage of the recovered radiolabel within the antigen-challenged ear site compared with the non-challenged ear. The results of this experiment (Table 2) confirmed the previously noted depression in the capacity to adoptively transfer CHS to previously irradiated recipients. The depression in edema generation was paralleled by a depression in the influx of radiolabeled lymphocytes into this site. Busulfan treated mice exhibited a depression in lymphocyte localization into the ear challenge site (and depressed CHS responsiveness) 1 month post-BMT. By 2 months, however, the ability of adoptively transferred effector lymphocytes to elicit edema and enter sites of antigen challenge was significantly better than their irradiated counterparts, and approached the response within normal age-matched (control) recipients.

DISCUSSION

The optimal preparative regimen for use in human BMT is a controversial issue, centered mainly around the marrow-

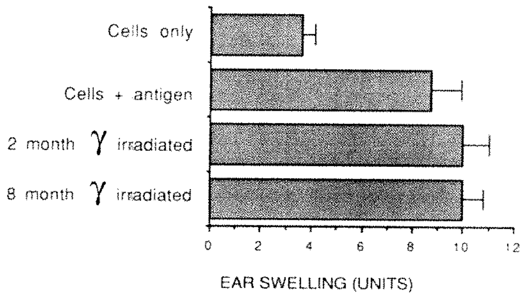

Fig 5. The assessment of CHS responses produced by coinjecting antigen and effector cells directly into the skin. TNBSO$_3$-haptenized normal spleen cells were produced by techniques described in the Materials and Methods section to serve as target cells. Normal mice were sensitized to 7% TNCB to provide an effector cell population. The peripheral lymph nodes of these mice were isolated and used to prepare a single cell suspension of effector lymphocytes. Both effector and target cells were suspended at 10^8 cells/mL, and mixed in a 1:1 ratio. A small volume (10 μL) of this combined cell suspension was injected into the right ear pinnae of irradiated recipients. The increment in right ear swelling (×10^{-4} inches) was measured at 48 hours to minimize any edema due to injection trauma. Similar injections into the ears of normal mice served to establish potential maximal edema generation by CHS effector cells in the presence of antigen. A group of mice that received an equivalent volume of media containing effector lymphocytes, but no antigen, served as a negative control.

Table 2. Localization of Adoptively Transferred CHS Effector Lymphocytes Into Cutaneous Sites of Antigen Deposition

Time Post-BMT	Ear Challenge	Ear Localization of Lymphocytes (%)	Ear Swelling (U)
Normal			
1 mo	+	3.2 ± 0.2	18.5 + 5.1
	−	0.1 ± 0.03	
2 mo	+	3.1 ± 0.6	15.0 + 3.0
	−	0.3 ± 0.2	
Irradiated			
1 mo	+	1.9 ± 0.4	0.3 + 0.2
	−	0.3 ± 0.1	
2 mo	+	1.2 ± 0.3	2.3 + 1.1
	−	0.1 ± 0.02	
Busulfan			
1 mo	+	1.5 ± 0.5	5.7 + 3.2
	−	0.02 ± 0.02	
2 mo	+	2.2 ± 0.5	11.5 + 0.8
	−	0.5 ± 0.3	

Normal donor mice were sensitized on two sequential days with DNFB as described in the Materials and Methods section. A single cell suspension was prepared from the peripheral lymph node lymphocytes of these animals. The sensitized effector lymphocytes were labeled for two hours with 2 μCi [^{125}IUDR] per 5 × 10^7 cells, followed by extensive washes. Groups of experimental mice that had been transplanted 1 or 2 months previously using either the busulfan or irradiation protocols were infused with 4 × 10^7 of these cells per recipient. Each recipient was then immediately challenged with 10 μl DNFB applied to the right ear. After 24 hours, the increment in ear swelling, comparing the challenged *v* the unchallenged ear, was established with a micrometer. This measurement is expressed in units ± SEM (10^{-4} in). The mice were subsequently killed and autopsied, and their organs (including each ear, individually) and carcass counted in a gamma counter to determine the relative distribution of cells. The percentage of sensitized lymphocytes localizing to the challenged and nonchallenged ear is shown.

ablative capacity[41] and acute toxicity of busulfan or irradiation-based regimens.[1,2] The long-term sequellae of either preparative regimen are not yet fully known.[42] We raised the possibility that irradiation-based protocols might cause long-term effects of the microvasculature of irradiated/transplanted mice when we noted protracted atrophy of the peripheral lymph nodes in these animals.[29] Since 95% of the lymphocyte content of peripheral nodes enters this tissue from the bloodstream, our observation implied that the anatomy or function of the lymphocyte-receptive endothelium of the lymph nodes (HEV) was, in some way, affected by the preparative regimen.

Acute exposure of microvascular endothelial cells to low doses of radiation (4.5 to 7.5 Gy) has been shown to produce increased vascular permeability, vasodilation, and intimal swelling, particularly within the skin.[43,44] These changes are believed to be reversible. Radiation-induced vascular fibrosis and decreases in capillary density have not generally been observed with doses <20 to 40 Gy.[45] Our previous studies in mice established that exposure of the lymph node microvasculature to relatively low doses of gamma-irradiation (>7.5 Gy) caused significant anatomic changes. Endothelial cell edema and microvascular occlusions were observed within areas of lymphocyte-receptive endothelium (HEV), which persisted for more than 6 to 8 months post-BMT. These histologic findings correlated with a depressed capacity for normal lymphocytes to enter irradiated lymph nodes from the bloodstream.[29] Similar changes were found on examination of lymph nodes derived from irradiated human BMT recipients up to 1 year post-BMT.[29] The lesions in both murine and human BMT-recipients were histologically distinct from previously described radiation-induced microvascular lesions.[43-45]

The current investigation established that busulfan had little effect on lymphocyte recirculation and HEV anatomy in mice. While irradiation caused apparent endothelial cell proliferation, intracellular edema, and microvascular occlusions, busulfan did not affect the appearance of HEV structures. These anatomic changes correlated with a depression in the ability of lymphocytes to localize into the lymph nodes of irradiated, but not busulfan-treated mice.

Since lymph nodes are believed to be important sites for antigen recognition and amplification of immune responses,[23,27] we hypothesized that changes in lymphocyte localization into these organs following BMT would be accompanied by demonstrable depressions in immune function. We compared the effects of busulfan and irradiation on the reconstitution of CHS responsiveness in transplant recipients. CHS is believed to be an anatomically restricted (compartmentalized) immune response that involves a circuit that Streilein has termed the skin-associated lymphoid tissue (SALT).[39] Lymphocyte tissue localization is believed to be necessary for this response. During the afferent limb (ie, antigen sensitization), the continual migration of T cells into peripheral nodes contributes to foreign antigen recognition and the development and clonal expansion of CHS-effector cell populations.[40] The antigen-sensitized effector lymphocytes are also believed to gain the capacity to localize selectively from the blood into sites of antigen challenge in the skin.[39,40]

Our studies demonstrated that CHS responsiveness recovered more quickly in busulfan-treated mice than in irradiated animals. The cause of the depression of CHS responsiveness in irradiated animals is difficult to interpret, since one step or multiple steps in the antigen sensitization and effector phases of the response could be affected by irradiation. Subsequent experiments established that one of the mechanisms that may contribute to a diminished CHS response in irradiated animals was the depressed capacity of lymphocytes derived from normal donors to localize into irradiated skin sites. Since proliferating cells were labeled in these studies, our results may reflect an irradiation-induced depression in the skin localization of the CHS effector cells. It should be remembered, however, that recruited, nonantigen-sensitized lymphocytes contained in the adoptively transferred population may also contribute to the cellular influx into the skin. The role of each cell population in the generation of the CHS response is as yet not fully characterized. Our preliminary studies appear to indicate that the ability of the recruited (nonantigen-sensitized) lymphocyte population to enter the skin is also significantly depressed following irradiation (unpublished data). Locally injected effector cells, on the other hand, appear to have a normal capacity to elicit skin edema, and are unaffected by the prior irradiation. This appears to exclude the possibility that irradiation alters the ability of the skin itself to respond to cellular and soluble stimuli required to elicit the CHS response.

The capacity of normal CHS effector cells to migrate into the skin of busulfan-treated recipients was also depressed at early times post-BMT, perhaps due to a transient effect of the drug on lymphocyte receptive endothelium. Two months after BMT, the capacity of CHS effector cells to enter the skin of busulfan-treated mice had recovered, unlike their irradiated counterparts. The two preparative regimens, therefore, appear to exhibit differing effects on the interaction of circulating normal lymphocytes with the skin microvasculature. Whether this difference is due to alterations in lymphocyte binding to endothelial cells or anatomic occlusions of vessels (as in irradiated peripheral lymph nodes) remains to be established. These experiments have, for the first time, correlated the lymphocyte tissue localization process and the restoration of normal immune function post-BMT.

There have been a number of human BMT studies[13,46-48] that have attempted to define the recovery of contact hypersensitivity following tranplantation. This immune response appears to be depressed for years following human BMT.[3,4] The majority of these studies were based on limited numbers of patients.[46-48] These reports also do not distinguish between de novo induction of contact hypersensitivity and cellular "memory" responses to recall antigens.[46-48]

Witherspoon et al recently reported the results of skin testing in 332 allogeneic and twin transplants.[13] These investigators found that skin test responses to recall antigens remained depressed for up to 4 years following transplantation. The de novo induction of CHS to DNFB was also assessed. This response appeared to recover 2 years or more after transplantation. No differences could be detected in the percentage of responding patients or in the timecourse of

CHS recovery between twin and allogeneic transplant recipients. The presence of acute or chronic graft-v-host disease, however, was found to significantly depress the frequency of positive skin test responses. Patients prepared with total body irradiation alone were hyporesponsive to DNFB sensitization for a more protracted period following transplantation than were patients treated with cyclophosphamide alone (an alkylating agent).[13] Patients pretreated with a combination of the two agents (presumably with attenuated doses of both) responded more like the cyclophosphamide pretreated group.[13] The findings of Witherspoon et al appear to support our conclusion that alkylating agents may be less detrimental to the recovery of CHS responses than total body irradiation, but caution must be used in extrapolating between the two studies. Human BMT patients represent a more complex system than our syngeneic murine transplantation model, and Witherspoon's patients were treated with a different set of preparative regimens than we used (with potentially differing toxicities). Despite these differences, similar mechanisms may contribute to the irradiation-induced depression in CHS responsiveness observed within both studies.

Other mechanisms are also likely to contribute to the depressed CHS responses seen in transplant recipients. Delayed T cell precursor proliferation and maturation in the posttransplant period may depress cellular immune function.[3,4,7,9] Depressions in the function of irradiated thymic epithelium may contribute to the delayed reconstitution of functional T cell subsets in secondary lymphoid organs.[49] Reconstitution of the T cell repertoire may not necessarily be complete following BMT. Relative imbalances in the number and functional capacities of helper and suppressor T cells may also develop and result in dominant suppression of immune responses.[9,10]

It is attractive to speculate that irradiation-induced depression of lymphocyte migration into the peripheral lymph nodes also contributes to the depressed recognition of antigens applied to the skin (ie, the afferent phase of the immune response). It is difficult, however, to establish this point experimentally. A number of distinct intracellular, intercellular, and cytokine-mediated processes are required for effective antigen sensitization and clonal expansion of effector lymphocyte populations. Dissecting the various elements of the afferent phase from each other in vivo is not a simple task. Experiments designed to estimate the relative efficiency of CHS effector cell generation within the peripheral nodes of irradiated v busulfan-treated mice are now in progress. If the number of CHS-effector cells within the nodes of irradiated animals is depressed, further studies will be necessary to establish the responsible mechanism(s).

We have intentionally used a simplified syngeneic transplantation protocol to avoid any potential interference with immunologic reconstitution by graft-v-host disease. The analysis of the marrow-ablative and immunologic consequences of single agents has allowed the comparison of their relative effects on microvascular anatomy, lymphocyte localization potential, and eventual immune reconstitution. This experimental model could easily be adapted for the evaluation of more complex preparative regimens. Such studies appear to be necessary to more accurately duplicate human BMT protocols. Most preparative regimens used in human BMT involve multiple, potentially interacting agents, to achieve host bone marrow ablation. Furthermore, immunomodulatory drugs such as methotrexate, azathioprine, cyclosporine A, and corticosteroids are often administered to allogeneic BMT recipients to decrease the frequency of graft-v-host disease. Each of these agents has the potential to interfere with lymphocyte localization into tissue. We have already demonstrated that corticosteroids act on endothelial cells and reversibly impair the ability of normal lymphocytes to enter peripheral lymph nodes and the skin following their parenteral administration to mice. Corticosteroids additionally appear to block CHS-effector cell functions following in vitro treatment of lymphocytes.[50] Further investigation of the effects of BMT-preparative regimens on the immunologic reconstitution of the host may lead to effective marrow-ablative regimens that minimize alterations in the lymphocyte recirculation process and allow rapid recovery of the immune system following transplantation.

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Platelet-Activating Factor Both Stimulates and "Primes" Human Polymorphonuclear Leukocyte Actin Filament Assembly

By Meir Shalit, Guissou A. Dabiri, and Frederick S. Southwick

The phospholipid inflammatory mediator, platelet-activating factor (PAF), can stimulate polymorphonuclear leukocyte (PMN) chemotaxis. Conversion of cytoplasmic actin from monomers to filaments is associated with PMN motile functions. Using the fluorescent actin filament stain nitrobenzodiaxole phallicidin, we have investigated PAF's effects on human PMN actin polymerization. Concentrations of PAF between 1×10^{-11} to 1×10^{-6} mol/L induced actin filament (F-actin) assembly. An optimal concentration of PAF ($1\text{-}5 \times 10^{-8}$ mol/L) induced a significantly lower rise in relative F-actin content (1.72 ± 0.07 SEM) than an optimal concentration (5×10^{-7} mol/L) of the chemotactic peptide FMLP (2.21 ± 0.06). Unlike FMLP (F-actin content: 1.25 ± 0.04 at five seconds), PAF stimulation was associated with a delay of more than five seconds (1.04 ± 0.01 at five seconds) before an increase in F-actin could be detected. F-actin concentration reached maximum levels by 30 to 60 seconds. Prolonged stimulation (20 minutes) with PAF was associated with two phases of polymerization and depolymerization. Like FMLP, the initiation of actin filament assembly by PAF required receptor occupancy, this reaction being totally blocked by the PAF receptor inhibitor, SKI 63-441. As evidenced by the lack of inhibition by nordihydroguaiaretic acid (5 to 20 μmol/L), the production of leukotriene B_4 was not required for the PAF-induced changes in F-actin. Like FMLP, PAF's ability to stimulate PMN actin polymerization was inhibited by pertussis toxin (.05 to 2.5 μg/mL) but not impaired by the addition of EGTA and/or the calcium ionophore A23187. Preincubation with 1×10^{-11} to 1×10^{-8} mol/L PAF for 2 to 60 minutes enhanced the rise in F-actin content induced by low concentrations of FMLP (5×10^{-12} to 1×10^{-10} mol/L) indicating that this phospholipid was capable of "priming" the PMN actin polymerization response.

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WHEN EXPOSED to a chemotactic gradient, polymorphonuclear leukocytes (PMNs) polarize and migrate. These motile functions require marked changes in the shape and consistency of the peripheral cytoplasm. Actin, the most abundant contractile protein found in the cytoplasm of PMNs, plays a critical role in controlling phagocyte motility.[1] This protein exists as 42,000 dalton monomers, G-actin, or can polymerize to form filaments, F-actin.[2] Actin filament formation could serve to increase the rigidity of the peripheral cytoplasm and mediate the shape changes associated with chemotaxis.[1,3] Unstimulated PMNs contain a high percentage of G-actin.[4,5] Stimulation with the synthetic chemotactic peptide f-met-leu-phe (FMLP) causes the rapid conversion of actin monomers to filaments.[4-10]

In addition to peptide chemotactic agents, such as FMLP, lipid inflammatory mediators such as platelet activating factor (PAF) may play an important role in activating PMN motility. This agent is synthesized and released by stimulated macrophages, platelets, and neutrophils.[11,12] In addition to being released, a high percentage of PAF synthesized by PMNs remains membrane bound.[13] PAF has been shown to induce PMN chemotaxis as well as degranulation and aggregation.[14,15] This agent has also been shown to induce PMN actin polymerization.[16-18]

We have used nitrobenzoxadiazole (NBD)-phallicidin, a fluorescent stain that binds specifically to F-actin[7,19] and flow cytometry to systematically examine the effects of this lipid chemotactic agent on neutrophil actin polymerization. We have found that stimulation with PAF, unlike FMLP, is associated with a five-second lag period before filament assembly begins. Prolonged exposure to this agent is associated with two phases of PMN actin polymerization and depolymerization. In addition to directly stimulating actin polymerization, PAF also can "prime" human PMNs, making the PMN actin polymerization response more sensitive to suboptimal concentrations of FMLP.

MATERIALS AND METHODS

Aprotonin, benzamidine, dimethyl sulfoxide (DMSO), EDTA, EGTA, FMLP, gelatin, HEPES, leupeptin, lysophosphatidyl choline, phenylmethyl-sulfonyl fluoride (PMSF), and triton X-100 were purchased from Sigma Chemical Company, St Louis. L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl (PAF), DL-α-lysophosphatidyl choline, γ-O-hexadecyl (lyso-PAF), A23187 were obtained from Cal-Biochem, San Diego. Bovine serum albumin was purchased from Reheis Chemical Company, Phoenix; pertussis toxin from List Biological Laboratory, Campbell, CA. Nitrobenzoxadiazole-phallacidin was obtained from Molecular Probes, Junction City, OR. The PAF antagonist SRI 63-441 was kindly supplied by C.W. Winslow of Sandoz, Hanover, NJ.

A modified Hank's buffer (138 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 5 mmol/L $NaHCO_3$, 0.64 mmol/L Na_2HPO_4 (pH 7.4), 0.66 mmol/L KH_2PO_4, 5.6 mmol/L glucose, 20 mmol/L HEPES) was used in all assays. $CaCl_2$, final concentration of 1 mmol/L, was added to cell suspensions ten minutes before cell stimulation unless otherwise noted in the text.

FMLP and A23187 (final concentrations 10 mmol/L) were dissolved in DMSO and kept frozen at −70°C until used. PAF and lyso-PAF were dissolved in modified Hank's solution containing

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0.25% BSA (final concentration 10 mmol/L) and stored at −70°C. Just before addition to cells, the stock solutions were diluted in modified Hank's solution. BSA could not be used in our experiments since this protein quenched NBD-phallacidin fluorescence. The PAF antagonist SRI 63-441 was dissolved in modified Hank's solution just before use.

Preparation of neutrophils. Six milliliters of venous blood were drawn from normal human volunteers into syringes containing 0.4 mL of 10% EDTA. RBCs were removed by hypotonic lysis, followed by centrifugation at 400 g for five minutes. Cells were resuspended in modified Hank's buffer and centrifuged at 400 g for five minutes. This procedure was repeated once and cells resuspended in modified Hank's to achieve a final concentration of 1.5×10^6 cells/mL. These preparations contained a mixture on average of 83 ± 1.4% (SEM) PMNs, 11 ± 1.7% lymphs and 5.1 ± 0.8% monocytes as determined by Wright stain (nine determinations). Platelets were not seen in any of our preparations. This method was developed to minimize handling of cells and reduce the likelihood of inadvertent PMN activation prior to chemotactic stimulation. On several occasions PMNs were purified by Ficoll/Hypaque which yielded >95% PMNs. Unstimulated F-actin content of PMNs tended to be higher in these preparations; however, the relative increase in PMN F-actin induced by PAF was comparable to cells purified by our other method. Cells were maintained at room temperature unless otherwise noted. All experiments were performed within three hours of venipuncture.

Neutrophil stimulation and fixation. Cell suspensions, 0.5 mL aliquots, (0.75×10^6 cells per sample) were preincubated for seven minutes at 25°C. Various concentrations of stimulants or antagonist, 0.5 mL, were added to each sample. The reaction was stopped by addition of 0.1 mL of 37% formaldehyde. Cells were incubated in fixative for 15 minutes at 25°C before staining.

NBD-Phallacidin staining. Staining was performed according to the method of Howard and Oresajo.[9] Briefly, 50 μL of modified Hank's buffer containing 3.46×10^{-6} mol/L NBD-phallacidin and 2.1 mg/mL lysophosphatidyl-choline were added to the fixed cell preparations, mixed and incubated for ten minutes at 37°C. Cells were then centrifuged at 12,000 g for one minute, supernatants removed, and pellets resuspended in 1.5 mL of modified Hank's solution.

Quantification of F-actin content by flow cytometry. Stained cells were filtered through nylon mesh and analyzed within two hours of staining in most cases. We have found that stained samples could be stored at 4°C in Hank's buffer for 16 hours without detectable differences in F-actin content as compared with the samples analyzed immediately after staining. The intracellular fluorescence was determined by an Ortho Spectrum III (Ortho Diagnostic Systems, Westwood, MA) flow cytometer equipped with an argon laser (488 emission, 500 mW output). Using the combination of low angle forward light scatter and 90° angle light scatter, lymphocytes, monocytes, remaining erythrocytes, and debris were excluded from analysis by appropriate gating. All intensities of cellular fluorescence were recorded on the linear scale ranging from 0 to 255 channels. Fluorescent histograms plotting cell number (vertical axis) *v* fluorescence channel (horizontal axis) were recorded for each sample. In all instances histograms yielded a normal distribution (the peak fluorescence channel corresponded to the mean fluorescence). An average of 5,000 to 10,000 PMNs were analyzed per sample. Relative PMN F-actin content was expressed as the ratio of mean fluorescence intensity of stimulated cells to mean fluorescence intensity of neutrophils in buffer. Unstimulated values were comparable when cells were incubated in modified Hank's buffer or 0.01% DMSO (the highest concentrations used in our experiments) in modified Hank's buffer.

Triton-insoluble cytoskeleton associated actin. A modification of the method described by White et al[6] was used. Human PMNs were purified by Ficoll/Hypaque and then suspended in modified Hank's buffer, centrifuged at 400 g and resuspended in the same buffer to achieve a final concentration of 1.5×10^7 cells per mL. Highly purified preparations of PMNs were required for this assay, since unlike FACS analysis that allows exclusive measurement of PMNs, the Triton-insoluble assay measures shifts in F-actin content of all cells in the solution. Five hundred microliters of the cell suspension was added to 55 μL of FMLP or PAF solution. The reaction was stopped by the addition of 65 μL of triton-stop solution containing 10% triton X-100, 7.5 mg% benzamidine, 4 mg% aprotinin, 1 mg% leupeptin, 1 mmol/L PMSF, 54 mmol/L Pipes, 22.5 mmol/L HEPES, 9 mmol/L EGTA, 1.8 mmol/L $MgCl_2$.

After mixing by two gentle inversions, the solution was allowed to sit for two minutes at 25°C, and then centrifuged for two minutes at 12,000 g in an Eppendorf table top centrifuge. The resulting pellet (the triton-insoluble fraction) was then solubilized in 100 μL of gel sample buffer containing 8 mol/L urea, 1 g% sodium dodecyl sulfate, 2 g% β-mercaptoethanol, 10 g% sucrose, 0.06 mol/L Tris-HCl, pH 6.8. The sample was next sonicated for 20 to 30 seconds using a Branson sonifier, model 200 with a tapered micro tip (Danbury, CT), output 3. Samples were then immediately boiled for five minutes and electrophoresed into 5% to 15% polyacrylamide slab gels. Triton-insoluble cytoskeleton-associated actin was quantified as previously described.

Statistics. The statistical significance of differences between means was determined using Student's *t* test.

RESULTS

The effects of varying concentrations of PAF on PMN F-actin content. Figure 1 demonstrates the effect of varying PAF concentration on neutrophil actin polymerization. A wide range of PAF concentrations 10^{-11} to 10^{-6} mol/L caused an increase in neutrophil F-actin content. A maximal relative F-actin concentration was observed at concentrations of 1-5 × 10^{-8} mol/L PAF. Above this concentration range a moderate decrease in F-actin content was observed.

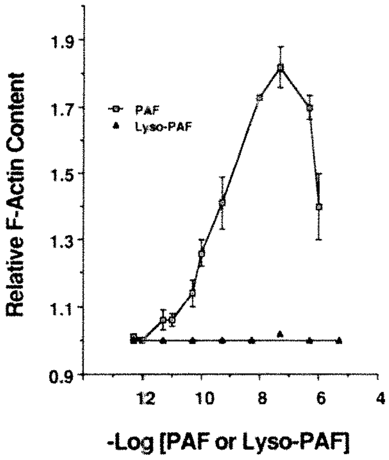

Fig 1. F-actin content as a function of PAF and Lyso-PAF concentration. 7.5×10^5 PMNs (similar numbers of cells were used for all experiments) were exposed to increasing concentrations of PAF or lyso-PAF for 60 seconds at 25°C. Cells were then fixed with 3.7% formalin, lysed, stained, and analyzed by FACS for relative F-actin content. The points represent the mean ± SEM of four to eight trials on separate days. Higher lyso-PAF concentrations (5×10^{-8}-5×10^{-6} mol/L) represent the mean of three determinations, lower concentrations represent values from one experiment.

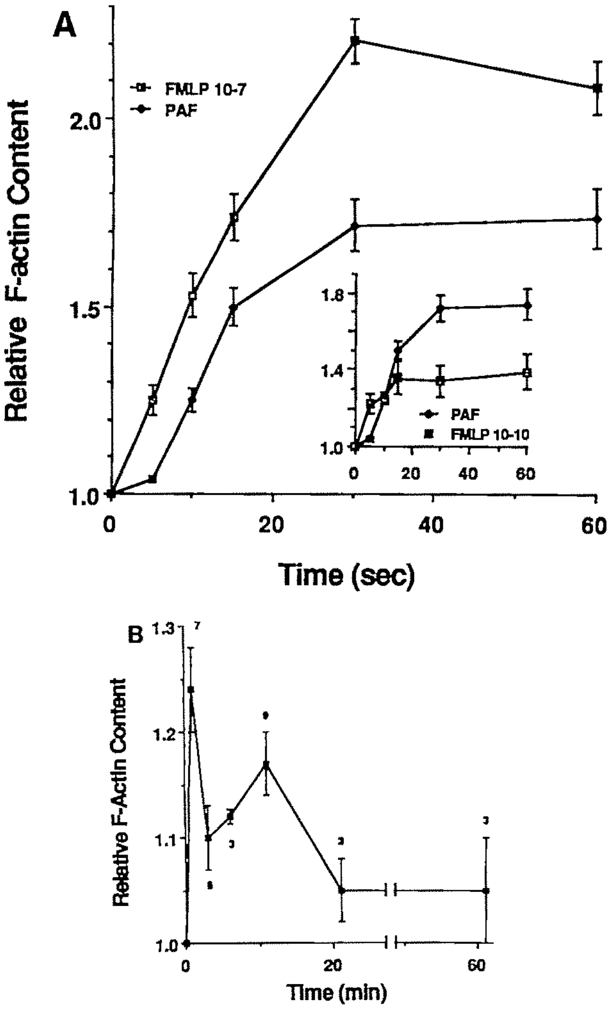

Fig 2. (A) Early time course of the changes in relative F-actin content in human PMNs exposed to optimal concentrations of PAF and FMLP. PMNs were exposed to 1 × 10^{-8} mol/L PAF (closed diamonds) or 5 × 10^{-7} mol/L FMLP (open squares) at time = 0 sec, at 25°C. At the indicated times cells were analyzed as described in Fig. 1. The points plotted represent the mean ± SEM of 13 (FMLP) and 8 (PAF) trials determined on separate days. Note the difference in F-actin content after five seconds stimulation with the two stimuli (FMLP 1.25 ± 0.04 *v* PAF 1.04 ± .01). This difference was statistically significant, ($P < .001$). Actin filament concentrations were also measured after five minutes stimulation with PAF and FMLP (respective values: 1.34 ± 0.05 and 1.74 ± 0.08 SEM, five determinations). Inset: F-actin content of PMNs stimulated with 1 × 10^{-10} mol/L FMLP (open squares) compared with PMNs stimulated with 1 × 10^{-8} mol/L PAF (closed diamonds). Note that after five seconds stimulation with this lower concentration of FMLP, F-actin content (1.22 ± 0.02 SEM, three determinations) was also significantly higher than after five seconds' stimulation with an optimal concentration of PAF ($P < .001$). (B) The effects of prolonged exposure of PAF on PMN actin filament content. PMNs were incubated at 25°C with 1 × 10^{-10} mol/L PAF and F-actin content measured at the times indicated. Bars represent the SEM, the numbers above the bars, the number of separate determinations.

Stimulation with the PAF analog lyso-PAF at concentrations of 5 × 10^{-13} to 5 × 10^{-6} mol/L failed to stimulate neutrophil actin assembly.

The kinetics of actin filament assembly following PAF stimulation. As shown in Fig 2A, exposure of human PMNs to an optimal concentration of PAF (1 × 10^{-8} mol/L) for various time periods resulted in a rise in F-actin content that reached a maximum value by 30 seconds. PAF's effects on PMN actin polymerization were delayed as compared with FMLP. After a five-second exposure to PAF there was no significant rise in F-actin content (F-actin content 1.04 ± .01 SEM, eight experiments) while FMLP (5 × 10^{-7} mol/L) induced a substantial increase in F-actin during the same period (relative F-actin concentration 1.25 ± 0.04 SEM, 13 experiments). Use of higher concentrations of PAF, up to 5 × 10^{-7} mol/L failed to shorten this delay period (five seconds; F-actin content, 1.03). Concentrations of PAF of 10^{-6} mol/L or higher could not be used, since as observed by other investigators,[20] these concentrations were associated with cell lysis. Addition of a low concentration of FMLP, 1 × 10^{-10} mol/L, was not associated with a delay in actin filament assembly. The initial rise in F-actin content after five seconds, 1.22 ± .05 SEM (three determinations), was nearly identical to that induced by 5 × 10^{-7} mol/L FMLP (Fig 2A inset).

Although the onset of actin filament formation was delayed, once this reaction began, the actin filament assembly rate associated with PAF was comparable to FMLP (4.4% increase in F-actin content/s *v* 4.8%/s for FMLP), and maximum F-actin concentrations were observed after a 30-second exposure to either stimulus. The peak concentration of F-actin induced by PAF was considerably lower than that induced by an optimal concentration of FMLP (1.72 ± 0.07, 10 experiments *v* 2.21 ± 0.06, 13 experiments). At each time point, F-actin content was significantly lower following PAF stimulation as compared with FMLP ($P < .05$). F-actin content following 30 seconds stimulation with optimal concentrations of the two agents was also measured using the triton-insoluble-cytoskeleton assay.[6] By this method PAF was also shown to induce a smaller rise in F-actin content as compared with FMLP (relative F-actin content: 1.6 *v* 2.0).

F-actin content rapidly decreased when PMNs were exposed to PAF for greater than one minute, reaching a nadir between 2 and 6 minutes, then actin filament concentration again increased reaching a second peak after approximately ten minutes. This reaction was followed by a second decline in F-actin content. Figure 2B shows the effects of prolonged exposure to 1 × 10^{-10} mol/L PAF; similar kinetics were observed when PMNs were exposed to 1 × 10^{-8} mol/L PAF for extended periods.

Effects of a PAF receptor antagonist (SRI 63-441), pertussis toxin, and NDGA on PAF-induced PMN actin filament assembly. The PAF receptor inhibitor (SRI 63-441) blocked neutrophil actin filament assembly induced by PAF, complete inhibition being observed at 1 × 10^{-6} mol/L, 50% inhibition at 1 × 10^{-7} mol/L, and no inhibition at 1 × 10^{-8} mol/L (Fig 3).

The role of G proteins in mediating PAF-induced actin

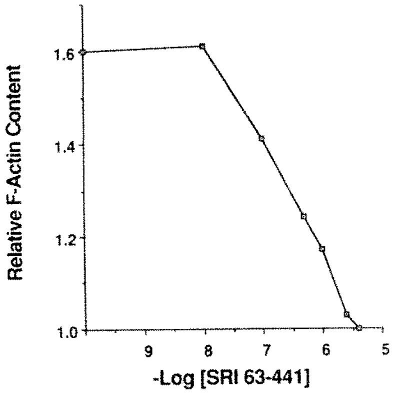

Fig 3. The effects of the PAF receptor antagonist SRI 63-441 on PAF stimulation of PMN actin polymerization. PAF 1 × 10^{-8} mol/L, was added simultaneously with varying concentrations of SRI 63-441. Samples were fixed, lysed, and stained after incubation at 25°C for 60 seconds. Values represent the mean of two determinations performed on separate days. The far left hand value (1.6) represents the F-actin content of PMNs stimulated in buffer.

filament assembly was examined using pertussis toxin. As observed with FMLP, incubation of cells for 90 minutes with 400 ng/mL of this agent caused a 50% to 60% inhibition of PAF-induced neutrophil actin assembly. Incubation with the same concentration of pertussis toxin for two hours resulted in 80% inhibition of both the PAF-and FMLP-induced rise in F-actin concentration. Higher concentrations of pertussis toxin (2.5 μg/mL) also resulted in marked inhibition of FMLP- and PAF-induced actin polymerization (Fig 4).

It has been suggested by some investigators that PAF activates PMNs by inducing endogenous production of leukotriene B_4.[21,22] To investigate whether production of this agent might be responsible for PMN actin polymerization, cells were preincubated with the lipoxogenase inhibitor, nordihydroguaiaretic acid (NDGA) before stimulation. Preincubation with 5, 10, and 20 μmol/L NDGA for five minutes at 37°C (concentrations previously shown to cause near total inhibition of leukotriene production by PAF[21-23]) failed to significantly inhibit the rise in F-actin content induced by PAF. A higher concentration (50 μmol/L) of this reagent inhibited both FMLP and PAF stimulation of actin polymerization and was associated with blebbing of the PMN peripheral cytoplasm, suggesting a more generalized toxic effect.

The effects of EGTA and the ionophore A23187 on PAF- and FMLP-induced neutrophil actin filament assembly. To assess the calcium requirement of PAF-induced neutrophil actin assembly, EGTA and the calcium ionophore A23187 were added to the incubation media. As shown in Fig 5, EGTA in the presence or absence of A23187 failed to inhibit PAF's effects on actin filament assembly. Addition of 1 mmol/L EGTA to the media also failed to inhibit the second rise in F-actin content observed after 20 minutes' exposure to PAF (Fig 2B). Similarly FMLP-stimulated PMN actin filament formation was not inhibited by addition of EGTA and/or A23187 to the buffer. In an additional experiment not depicted in Fig 5, PMNs were preincubated with EGTA for 20 minutes followed by exposure to A23187 for four rather than two minutes to further deplete intracellular calcium stores. This condition also failed to inhibit PAF- and FMLP-induced rises in F-actin content. Values were comparable to those depicted in column d of Fig 5. As observed by other investigators,[24] A23187 in the presence or absence of EGTA caused moderate increases in F-actin concentration (1.33 ± 0.14 and 1.59 ± 0.06, respectively, after two minutes exposure, three determinations). A23187 alone, however, had no significant effect on

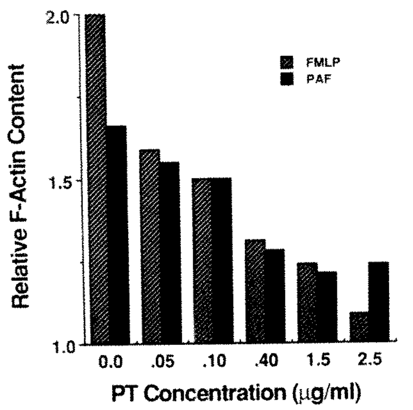

Fig 4. The effects of pertussis toxin (PT), on PAF and FMLP stimulation of PMN actin polymerization. PMNs were incubated with increasing concentrations of PT for 90 minutes at 37°C. Cells after being washed once in buffer were stimulated with PAF 1 × 10^{-8} mol/L or FMLP 5 × 10^{-7} mol/L for 60 seconds.

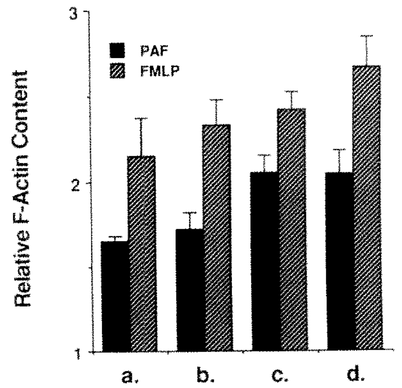

Fig 5. The effects of EGTA and the calcium ionophore A23187 on PAF and FMLP stimulation of PMN actin polymerization. PMNs were incubated at 25°C (a) in buffer containing 1 mmol/L CaC_2, (b) in buffer containing 1 mmol/L $CaCl_2$ and 5 × 10^{-7} mol/L A23187 for two minutes, (c) in buffer without added calcium plus 1 mmol/L EGTA for 20 minutes, or (d) in EGTA buffer for 20 minutes followed by the addition of A23187 for two minutes. PMNs were then stimulated with 2.5 × 10^{-8} mol/L PAF or 5 × 10^{-7} mol/L FMLP for 60 seconds at 25°C. Samples were analyzed as described in Fig 1. Values represent the mean ± SEM of experiments performed on three different days. The differences in F-actin content induced by PAF or FMLP under these four conditions were not statistically significant, with the exception of condition (d). The FMLP induced rise in F-actin content was significantly higher under these conditions when compared with PMNs stimulated with FMLP in buffer (a)(P = .014).

Table 1. Percent Increase in Relative F-Actin Content After 60 Second Stimulation With Various Concentrations of PAF or FMLP Alone and in Combination

[FMLP] (mol/L)	10^{-8}	10^{-9}	10^{-10}	10^{-11}	None
[PAF] (mol/L)					
10^{-8}	117 ± 23 (2)	—	98	—	70 ± 10 (2)
5×10^{-10}	—	108	—	—	62
$5\text{-}10 \times 10^{-11}$	—	81 ± 14 (2)	52 ± 11 (4)	—	28 ± 3 (6)
$1\text{-}10 \times 10^{-12}$	—	31	37 ± 21 (4)	21 ± 9 (3)	3 ± 1 (8)
None	119 ± 11 (2)	36 ± 5 (4)	11 ± 3 (9)	9 ± 3 (3)	0 (17)

PAF and FMLP were added simultaneously to 7.5×10^6 PMNs and cells fixed after 60 seconds. F-actin content was measured as described in Fig 1 and Materials and Methods. Numbers in parentheses represent the number of separate determinations. Values are percentages ± SEM.

PAF- or FMLP-induced neutrophil actin polymerization (Fig 5, column b).

Combined stimulation with PAF and FMLP As shown in Table 1, when PMNs were exposed to low concentrations of PAF and FMLP simultaneously, the F-actin content increased to levels higher than that induced by either stimulus alone. The resulting rise in F-actin content was generally equivalent to or above the sum of the rise in F-actin content induced by the individual chemotactic stimuli. This additive effect was seen when concentrations of FMLP of 7.5×10^{-10} to 1×10^{-11} mol/L were combined with 1×10^{-8} to 1×10^{-12} mol/L concentrations of PAF. Use of optimal concentrations of FMLP as well as PAF failed to stimulate increases in F-actin content above that induced by FMLP alone.

Preincubation of PMNs with PAF (1×10^{-10} mol/L) for 2, 5, 10, 20, and 60 minutes also enhanced F-actin production in response to a low concentration of FMLP (5×10^{-10} mol/L). At each time point the rise in F-actin was greater than the sum of the two individual stimuli (data not shown). As shown in Table 2, preincubation of PMNs with varying concentrations of PAF (1×10^{-11} to 1×10^{-8} mol/L) for 60 minutes resulted in an enhanced F-actin response to low concentrations of FMLP (5×10^{-12} to 1×10^{-10} mol/L). F-actin content was 20% to 200% greater than the sum of rises in F-actin content induced by the individual stimuli. Monocyte contamination did not appear to play a role in PAF "priming," since PAF preincubation of PMNs purified by Ficoll/Hypaque resulted in comparably greater than additive responses to low concentrations of FMLP (data not shown). Preincubation of PMNs with FMLP (5×10^{-11} to 5×10^{-10} mol/L), rather than PAF, for 60 minutes followed by addition of a higher concentration of FMLP for 60 seconds (1×10^{-10} to 1×10^{-9} mol/L) was associated with a less than additive rise in F-actin content in all instances (Table 3). The combined stimulus resulted in an F-actin content that was 10% to 62% below the sum of the individual stimuli.

DISCUSSION

Human and rabbit PMNs rapidly increase their F-actin content after being stimulated with FMLP. Under optimal conditions this chemotactic stimulus increases F-actin content by approximately twofold within 30 seconds. Other agents have also been shown to stimulate PMN actin polymerization including leukotriene B_4, phorbol myristate acetate, and arachidonic acid.[16] PAF has also been shown by the triton-insoluble cytoskeleton assay to induce a rise in the F-actin content of rabbit PMNs.[16,17] The fluorescent F-actin stain, NBD-phallicidin, has recently been used to measure F-actin content in PMNs.[7] Using this assay we have systematically compared the individual effects of PAF and FMLP on human PMN actin filament assembly and have also investigated how these two agents in combination may affect in vivo PMN actin polymerization. The NBD-phallicidin assay makes possible careful kinetic analysis of PMN actin filament formation and also allows reproducible quantitation of relative F-actin concentration.[9] These investigations have demonstrated several differences between FMLP and PAF.

First, on examining the kinetics of the PAF-induced rise in F-actin content, we found a brief but reproducible lag period of five seconds between the time of stimulation and the initial increase in F-actin content. This delay in actin polymerization was not observed after stimulation with FMLP even

Table 2. Percent Increase in Relative F-Actin Content After 60 Minutes Stimulation With Varying Concentrations of PAF Alone, After 60 Seconds Stimulation With Varying Concentrations of FMLP Alone and After 60 Minutes Stimulation With PAF Followed by 60 Seconds Stimulation With FMLP

[FMLP] (mol/L)	10^{-10}	5×10^{-11}	5×10^{-12}	None
[PAF] (mol/L)				
10^{-8}	54 ± 8 (+50)	42 ± 1 (+27)	24 ± 2 (+20)	18 ± 1
10^{-9}	44 ± 5 (+38)	37 ± 3 (+28)	38 ± 4 (+138)	14 ± 2
10^{-10}	35 ± 5 (+35)	31 ± 3 (+35)	30 ± 8 (+200)	8 ± 3
10^{-11}	39 ± 9 (+95)	30 ± 2 (+76)	11 ± 1 (+175)	2 ± 2
None	18 ± 4	15 ± 3	2 ± 1	

Numbers in parentheses represent the percent rise above the sum of the two individual stimuli: % F-actin content above additive = 100 × [F-actin content of PMN receiving combined stimuli − (F-actin content of PMN stimulated with FMLP × 60 + F-actin content after PAF × 60 min)]/(F-actin content after FMLP + F-actin content after PAF). Values are percentages ± SEM of three to seven separate determinations.

Table 3. Percent Increase in Relative F-Actin Content After 60 Minutes Stimulation With Varying Concentrations of FMLP Alone, After 60 Seconds Stimulation With Varying Concentrations of FMLP Alone and After 60 Minutes Stimulation with FMLP Followed by 60 Seconds Stimulation With a Second Higher Concentration of FMLP

[FMLP] (mol/L; 60 sec)	10^{-9}	5×10^{-10}	1×10^{-10}	None
[FMLP] (mol/L; 60 min)				
5×10^{-10}	66 (−31)	—	—	38
1×10^{-10}	61 (−12)	47 ± 9* (−10)	—	11 ± 2
5×10^{-11}	57 (−20)	31 ± 7 (−42)	13 ± 6 (−62)	13
None	58	41 ± 2	21 ± 7	

Numbers in parentheses represents the % below the sum of the two individual stimuli: % F-actin content below additive = 100 × [F-actin content of PMN receiving combined stimuli − (F-actin content of PMN stimulated with FMLP × 60 s + F-actin content after PAF × 60 min)]/(F-actin content after FMLP + F-actin content after PAF).

*Values are percentages ± SEM of three separate determinations; other experiments represent the mean of two separate determinations.

when low concentrations of FMLP were used to stimulate actin filament assembly. Stimulation with higher concentrations of PAF did not shorten the time of initial actin filament assembly, therefore a delay in binding of PAF to PMN membrane receptors seemed an unlikely explanation for our findings. Such a lag period might be present if PAF's effects on actin filament assembly were mediated by the production of a second compound, such as leukotriene B_4. However, preincubation of PMNs with the lipoxegenase inhibitor, nordihydroguaiaretic acid (NDGA), a condition that should block leukotriene production,[21-23] did not affect PAF-induced actin polymerization. When human PMNs bind PAF they rapidly metabolize this agent.[25] We cannot exclude the possibility that the observed lag period represents the time required to metabolize PAF to a more active byproduct.

Not only the early kinetics of polymerization, but also the time course of depolymerization may differ from FMLP. Prolonged incubation with PAF was associated with two phases of polymerization and depolymerization. The initial rapid rise in F-actin content was followed by a decrease in F-actin over the ensuing two to six minutes. Depolymerization was then followed by a moderate second rise in F-actin content that in turn was followed by a second depolymerization phase. With the exception of Sklar et al,[10] who observed two phases of polymerization and depolymerization during the first two minutes of oligopeptide stimulation, other investigators have described a single abrupt rise in F-actin content followed by a single phase of depolymerization following FMLP stimulation.[6,9] The cause of this second rise in F-actin content is not clear, but could be the result of PMN aggregation or the production of second mediator that in turn induces a second actin polymerization reaction.

There are also a number of similarities between these two chemotactic agents. Receptor inhibitor studies indicate that PAF, like FMLP, requires receptor occupancy to induce PMN actin polymerization.[10] The marked inhibitory effects of pertussis toxin on human PMNs suggest that as observed in rabbit PMNs,[24] both chemotactic agents require functioning G-proteins[26] to stimulate actin polymerization.

Neither PAF nor FMLP required extracellular calcium to induce increases in F-actin concentration. This finding suggests that the large influx of ionized calcium generally associated with PAF[24,27] and FMLP[28] stimulation is not required for actin filament assembly. The effects of combined treatment of PMNs with EGTA and A23187 are complex and variable; therefore, no firm conclusions can be drawn from our experiments concerning the importance of intracellular calcium in mediating PAF- and FMLP-induced actin filament assembly. These experiments do suggest that these two classes of chemotactic agent may function similarly, since this treatment failed to inhibit actin filament assembly induced by either reagent.

In addition to acting directly to induce neutrophil aggregation, degranulation, superoxide production, chemotaxis, and actin polymerization, PAF can regulate neutrophil responses to other stimuli. PAF can desensitize the PMN aggregation response to leukotriene B_4.[21] When combined with opsonized zymosan, PAF induces an additive rise in superoxide production.[29] When combined with FMLP, PAF synergistically enhances the oxidative response. Maximum enhancement is observed when optimal oxidative concentrations of both chemotactic agents are used (10^{-7} mol/L FMLP and 10^{-8} mol/L PAF).[30]

Our studies demonstrated that PAF also is able to modulate PMN actin responsiveness to FMLP. However, unlike the oxidative response, PAF only enhanced the effect of suboptimal FMLP concentrations. Simultaneous addition of or preincubation for up to 60 minutes with PAF caused an enhanced response to FMLP. Particularly after prolonged preincubation with PAF, the combined effect of these two chemotactic agents on F-actin content was consistently greater than the sum of the effects of two individual stimuli. Preincubation with FMLP followed by exposure to a second higher concentration of FMLP, on the other hand, resulted in a less than additive response. These findings suggest as observed for oxidative metabolism, that PAF may act as a "priming" stimulus[31] for FMLP. Although these two chemotactic agents bind to separate receptors, they may have common intermediate biochemical pathways serving to induce PMN actin filament assembly. PAF could serve to activate one or more proteins responsible for this reaction, a condition that might augment PMN actin assembly in response to a second stimulus. It is also possible that PAF stimulation may increase the number and/or affinity of FMLP receptors.

Understanding how these two chemotactic agents affect actin filament assembly alone and in combination are of importance in understanding the in vivo inflammatory response, since both of these chemotactic agents may be present in inflammatory exhudates and PAF may be synthesized and retained on the membrane surface of stimulated PMNs.[13] The present findings suggest that the lipid chemotactic agent, PAF, not only can induce PMN actin filament assembly, but may also serve to make the PMN actin response more sensitive to low concentrations of peptide chemotactic agents like FMLP.

ACKNOWLEDGMENT

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Cytogenetic Studies and In Vitro Colony Growth in Patients With Mastocytosis

By Birgitta Swolin, Stig Rödjer, and Gösta Roupe

Cytogenetic analysis of bone marrow cells and in vitro growth for bone marrow granulocytic-macrophage stem cells have been performed in 13 patients with mastocytosis, six with systemic mastocytosis, and seven with urticaria pigmentosa. Clones with chromosome abnormalities were found in five patients. The number of clusters and/or colonies after seven days in culture was increased in seven patients, compared with the growth in a control group. Three patients with chromosome abnormalities showed an abnormal growth pattern, yet exhibited normal peripheral blood values. Two patients with systemic mastocytosis had clones with chromosome abnormalities and some abnormal hematological values. The proportion of patients with chromosome abnormalities and an abnormal growth pattern was higher among these patients with mastocytosis than in healthy control subjects. These results may be of interest when discussing the origin of mast cell disorders and indicate an association with the myeloproliferative disorders.

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MASTOCYTOSIS is a rare disorder with manifestations either in the skin, as urticaria pigmentosa, or as a generalized form, systemic mastocytosis, or even as mast cell leukemia. Anemia, leukocytosis, thrombocytopenia, or eosinophilia may be present.[1] An increased number of mast cells in the bone marrow and lesions with varying numbers of lymphocytes, eosinophils together with mast cells, may be found.[2]

Certain patients with mastocytosis can initially be diagnosed as having or may later develop a myeloproliferative disorder.[3,4] Many patients with myeloproliferative diseases have chromosome abnormalities in bone marrow cells[5]; however, cytogenetic analysis has been reported in only a few cases with mastocytosis and normal karyotypes were then observed.[4,6,7] The in vitro colony-forming capacity of bone marrow granulocytic-macrophage stem cells can also show an abnormal growth pattern in myeloproliferative disorders. Animal cell culture studies indicate that the mast cell may be of bone marrow origin.[8] Due to these findings it would be of interest to investigate patients with mastocytosis with techniques that have revealed abnormalities in the myeloproliferative disorders. This paper reports on cytogenetic studies and bone marrow colony growth in 13 patients with mast cell disease. To our knowledge, no systematic studies have previously been carried out.

MATERIAL AND METHODS

Patients. Thirteen adult patients (median age 40 years) with mastocytosis participated, after informed consent, in the present study. Clinical data are presented in Table 1. The six patients (four males, two females) with systemic mastocytosis were 30 to 69 years of age (median 58 years) with a disease duration varying from 2 to 27 years (median 5.5 years). The age ranged between 19 and 60 years (median 28 years) for the seven patients (two males, five females) with urticaria pigmentosa and was lower than for patients with systemic mastocytosis. The disease duration was also shorter varying from 1 to 5 years (median 3 years).

The diagnosis of the skin disease was based on clinical criteria for urticaria pigmentosa, ie, multiple reddish-brown maculae that urticate after rubbing. The extension of the skin disease was calculated from the number of macular skin lesions, which was estimated as small, moderate, or large. The severity of the pruritus was estimated by each patient. None of the patients had a history of flush attacks, headache, or decreasing BP.

The clinical diagnosis was confirmed in all the patients by histological examination of punch biopsies from hyperpigmented macular spots from the abdominal skin. The diagnosis of systemic mastocytosis was based on direct demonstration of an increased number of mast cells in the bone marrow section and an increase in the urinary excretion of methylimidazole acetic acid.[2] Patient no. 6 with systemic mastocytosis had an increased number of mast cells in the bone marrow but normal excretion of methylimidazole acetic acid in urine. Splenomegaly was found in three patients (Table 1).

Control group. For comparison, the in vitro bone marrow colony growth capacity for granulocytic-macrophage stemcells was studied in nine subjects (seven males and two females) aged 23 to 70 years with a median age of 39 years, after informed consent. These healthy subjects had normal peripheral blood values and bone marrow morphology.

Sampling procedure. Bone marrow (1 to 2 mL) from the sternum or iliac crest was aspirated. The cells were suspended in 3 mL McCoy's medium (Flow Laboratories) containing 125 IU heparin (Kabi Vitrum) for colony growth and the marrow was suspended in 6 mL McCoy's medium for cytogenetic analysis.

The assay of colony formation was measured by using a two-layered semisolid, agar culture system described by Pike and Robinson.[13] Placenta extract was used as source of the colony-stimulating factor, according to Burgess et al,[14] instead of using mononuclear cells in the feeder layer. The top layer was seeded with 1×10^5 mononuclear cells per milliliter per dish. The cells were isolated on Isopaque-Ficoll. The cultures were incubated at 37°C, in a humified atmosphere of 5% CO_2 in air. Four dishes for each culture were used as for unstimulated controls, the mean value was given. After seven and ten days the dishes were examined with an inverted microscope. Aggregates of more than 40 cells were defined as colonies, 21 to 40 cells as macroclusters, and three to 20 cells as microclusters.[15] Under these conditions the highest value for colonies was attained on day 10 and the colony growth reached a plateau in the amount of placenta extract used. Growth was linear for up to ten times the cell concentration used. The reproducibility was good and tested from two sequential cultures in six subjects.

In the last six patients examined, the dishes were fixed and stained with May-Grünwald-Giemsa to evaluate the morphology of the

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Table 1. Clinical Findings in 13 Patients With Mastocytosis

Patient No.	Age at Examination (y)/Sex	Duration of Disease (y)	No. of Macular Skin Lesions	Pruritus	Spleen Area Scintigraphy (cm^2)*	Increased No. of Mast Cells in Bone Marrow Section†	U-MeImAA (mg/24 h)‡	Clinical Diagnosis
1	69/M	2	Small	Mild	195	Yes	58.2	SM
2	63/F	27	Large	Mild	Normal	Yes	48.4	SM
3	44/F	20	Large	Moderate	Normal	Yes	22.8	SM
4	30/M	5	Moderate	Moderate	Normal	Yes	7.1	SM
5	56/M	6	Large	No	110	Yes	4.9	SM
6	60/M	3	Moderate	Mild	104	Yes	3.3	SM
7	39/F	1	Moderate	No	Normal	No	2.5	UP
8	40/F	3	Small	Moderate	Normal	No	1.9	UP
9	19/F	5	Small	No	Normal	No	1.9	UP
10	60/F	4	Moderate	No	Normal	No	1.8	UP
11	25/M	5	Large	Moderate	Normal	No	1.8	UP
12	28/M	1.5	Small	Moderate	Normal	—	1.6	UP
13	19/F	1	Small	No	Normal	No	1.0	UP

Abbreviations: SM, systemic mastocytosis; UP, urticaria pigmentosa.

*Scintigraphic examination of the spleen was performed according to Larsson and Nelp in 1966.[9] Reference value <81 cm^2.[10]

†See Ridell et al.[2] The upper limit of mast cells for reference subjects is 71/mm^2.

‡U-MeImAA (urine methylimidazole acetic acid) was measured by high performance liquid chromatography as described by Granerus,[11] Granerus and Wass.[12] Reference values – 1.2 to 3.8 mg/24 h.

cells. The enumerated colonies and clusters included neutrophils, macrophages, eosinophils, and mast cells, as determined by the morphological appearance with this staining.

Chromosomes from bone marrow cells were prepared with conventional techniques. When no evaluable metaphases could be obtained in direct preparations, marrow cells were also studied after 48 hours in culture. G- and Q-banding was used.[16,17] Chromosome identification and karyotypes were classified in accordance with the International System for Human Cytogenetic Nomenclature,[18] ie, a clone was defined as at least two cells with the same numerical and/or structural abnormality.

Routine laboratory techniques were used for the determination of peripheral blood parameters. Plasma cobalamines (B_{12}) and folates concentrations were assayed with microbiological techniques.

RESULTS

The laboratory results are presented in Table 2. The hemoglobin concentration was normal in all patients except two (cases no. 1 and 2) with systemic mastocytosis (Table 2). The leukocyte counts were normal in all patients. Differential leukocyte count showed no significant abnormality; however, slight eosinophilia was found in patients no. 2 and 3. Patient no. 1 exhibited an increased platelet number, while all other patients had normal levels. All patients had serum iron and total iron binding capacity within reference values. Patients no. 1 and 2 exhibited an increased plasma B_{12} concentration and none of the patients had been given drugs containing B_{12}. Plasma and blood folates levels were normal in all patients.

The cytogenetic analysis of the bone marrow cells revealed clones with chromosome abnormalities in five patients (Table 3, Fig 1). Clones were present only to a minor extent, except in patient no. 8, and were revealed in three patients after a 48-hour culture. The type of abnormality was 5q−, 9p+, 11q−, +13 and −16, +mar in each patient. In patient no. 1 a loss of one no. 16 was seen and it cannot be excluded that the marker derives from no. 16. We regard the deletions in patients no. 2 and 6 as interstitial deletions. Patient no. 8 had some extra unidentified material on the short arm of

Table 2. Laboratory Results in 13 Patients With Mastocytosis

Patient No.	Hb g/L	WBC $\times 10^9$/L	Eosinophils $\times 10^9$/L	Platelets $\times 10^9$/L	S-Fe µmol/L	TIBC µmol/L	S-B_{12} pmol/L
1	110	8.9	0.14	550	20	55	1800
2	97	7.5	0.52	149	10	32	955
3	126	8.2	0.73	262	10	54	225
4	164	4.7	0.22	213	22	37	299
5	131	4.8	0.03	177	13	55	230
6	138	5.3	0.10	268	18	42	351
7	119	5.2	0.14	212	14	49	201
8	115	8.9	0.30	224	24	49	220
9	137	3.7	0.15	256	20	52	284
10	126	6.0	0.11	321	12	54	292
11	158	6.4	0.10	349	13	46	436
12	155	5.6	0.15	266	30	58	356
13	123	5.0	0.11	240	28	65	499
Reference values	(>110)	(3.5-10)	(0.06-0.3)	(150-350)	(10-30)	(43-90)	(130-740)

Table 3. Results of Cytogenetic Analyses and In Vitro Cultures for Granulocytic-Macrophage Progenitor Cells From Bone Marrow in Patients With Mastocytosis

				Number of Clusters and Colonies for CFU-GM/10^5 Cells			
				7 d			10 d
Patient No.	Bone Marrow Karyotype	No. of Cells Karyotyped	Proportion of Abnormal Clone (%)	Microcluster	Macrocluster	Colonies	Colonies
1	46,XY/46,XY, −16, +mar	28	21	208	102	11	14
2	46,XX/46,XX,del(5)(q21q31)	25*	13	252	112	40	48
3	46,XX	13	0	140	177	34	54
4	46,XY	11	0	359	324	303	242
5	46,XY	14	0	373	260	271	169
6	46,XY/46,XY,del(11)(q13q21)	11*	18	1029	343	75	45
7	46,XX	14	0	268	220	256	132
8	46,XX,9p+/46,XX	24	83	362	339	178	92
9	46,XX/46,XX+G/47,XX,+13	11*	27/18	231	229	438	240
10	46,XX	8	0	356	122	102	44
11	46,XY	11	0	427	13	0	4
12	46,XY	12	0	179	154	46	80
13	46,XX	14	0	361	331	52	86
Patients (n = 13), median (range)				356 (140-1029)	220 (13-343)	75 (0-438)	80 (4-242)
Control group (n = 9), median (range)				344 (173-907)	126 (76-267)	54 (1-220)	47 (22-168)

*Prepared after 48-h culture.

chromosome No. 9, which did not seem to be a pericentric inversion, however C banding was not performed. The extra G chromosome in patient no. 9 bore the closest resemblance to a no. 22. The other eight patients exhibited normal karyotypes. All patients had a normal karyotype in phytohemagglutinin stimulated peripheral blood cells.

Patients no. 1 and 2 with an abnormal karyotype had both anemia and elevated plasma B_{12}-concentrations.

The colony growth capacity in all the patients examined showed a median value of 356 (range 140 to 1,029) on day 7 for microclusters, 220 (range 13 to 343) for macroclusters, and 75 (range 0 to 438) for colonies (Table 3). On day 10, the median value was 80 for colonies (range 4 to 242). No obvious difference could be observed between the patients with systemic mastocytosis and those with urticaria pigmentosa. In comparison with the control group the number of colonies was nearly twice as numerous while the cluster counts were of the same order of magnitude. The largest number of clusters was reached after a seven-day culture. An increased number of clusters was found in patients no. 4, 6, 8, and 13. The number of colonies reached a peak after seven days of culture for seven patients, four of whom showed an increased number in comparison with the control group. Six patients had a larger number of colonies on day 10, but all had values within the reference values. Only patient no. 11 had defective colony growth.

The morphology in May-Grünwald-Giemsa stained clusters and colonies could be evaluated in patients no. 1, 3, 4, 7, 9, and 13. All patients exhibited cells with features similar to mast cells, especially with regard to the cell size and the

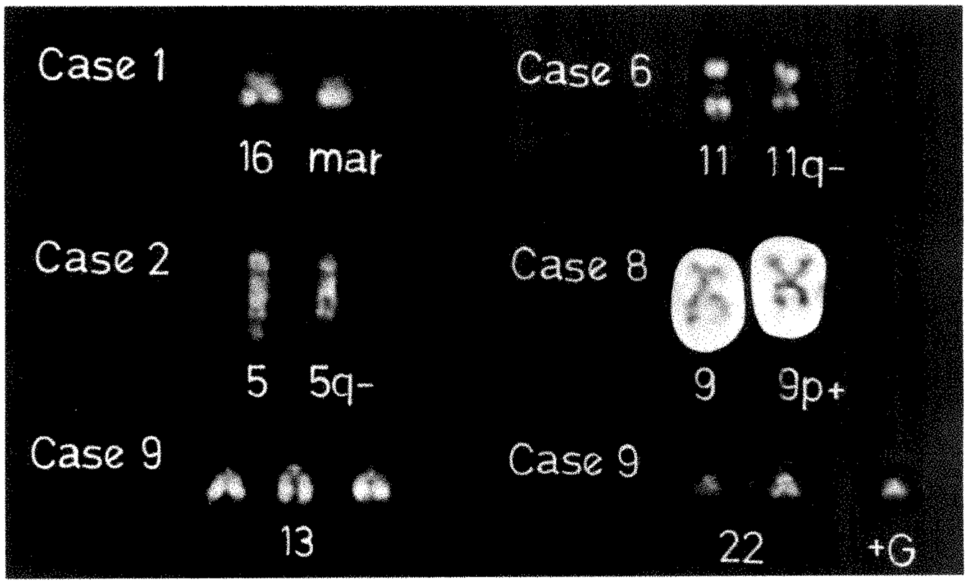

Fig 1. Bone marrow chromosomes, pairs with abnormalities in patients no. 1, 2, 6, 8, and 9.

heavy content of basophilic granules; however, further evaluation is necessary. Three of the five patients with chromosome abnormalities showed an abnormal in vitro growth pattern for granulocytic-macrophage stem cells.

DISCUSSION

Disorders with mast cell proliferation include a wide variety of manifestations, from benign infiltration of the skin, urticaria pigmentosa, to the malignant course seen in mast cell leukemia.[19] An intriguing connection between both myelo- and lymphoproliferative disorders has been discussed in case reports.[3,4]

This is the first report on a small series of patients with mastocytosis investigated by cytogenetic analysis. Chromosome abnormalities in bone marrow cells were found in 36% of the patients, which is a higher proportion than in healthy subjects.[5,20] In myeloproliferative disorders the incidence of abnormalities varies from 85% in patients with chronic granulocytic leukemia with the Ph^1 chromosome to ~15% in patients with polycythemia vera close to the diagnosis.[5,20,21] Some patients with chronic myeloid leukemia develop basophilia, and in a series[22] of patients with chronic myeloid leukemia the chromosome abnormality iso(17q) in addition to the Ph^1 abnormality, was related to high values of basophils. This abnormality, however, was not found in the present series of patients with mastocytosis. The abnormalities 5q− and 11q− found in two patients are also common abnormalities in myeloid disorders and in myelodysplastic syndromes.[23,24]

In seven patients, the in vitro colony growth pattern of bone marrow cells showed an increased number of clusters and/or colonies similar to findings recently reported by Denburg et al.[25] They found an increased number of colony-forming units in peripheral blood from patients with systemic mastocytosis. This pattern is more like the pattern found in chronic myeloid leukemia,[26] than that found in myelodysplastic syndrome with defective or no colony forming capacity.[15,27] An increased number of histamine positive granulocytic colonies from blood has been found in patients with systemic mastocytosis and chronic myeloid leukemia.[28] It would have been desirable to identify the cell type that contained the chromosome abnormalities. However, techniques for such identification were not available to us.

The occurrence of chromosome aberrations or increased growth capacity was similar in patients with urticaria pigmentosa and systemic mastocytosis. There was no obvious relationship between the cytogenetic results and the colony growth seen in this small series. However, three patients had both an abnormal karyotype and abnormal growth capacity, yet they still exhibited normal peripheral blood values.

All the patients with urticaria pigmentosa had normal hemoglobin, leukocyte, and platelet concentrations, as has been described in earlier reports.[1] In the group with systemic mastocytosis, one patient had anemia and thrombocytosis, and another had anemia and eosinophilia. These findings are in agreement with those in the reports on larger numbers of patients.[1,29] These two patients also had clones with chromosome abnormalities, as well as an increased plasma B_{12} concentration, a common finding in myeloproliferative disorders.

Bone marrow cells from our patients with mastocytosis showed chromosome aberrations and an abnormal in vitro colony growth similar to findings in myeloproliferative disorders and thus supports the idea of an association between these disorders. The hypothesis that mast cells derive from a hematopoietic origin is supported by observations obtained with bone marrow mast cells, grown in long-term culture from a patient with mastocytosis.[7] An increased number of bone marrow mast cells has been reported in preleukemic syndromes.[30] Experimental data suggest that the production of granulocyte-macrophage colony stimulating factor can be activated in tumorigenic mast cell lines derived from Abelson murine leukemia virus-transformed hematopoietic cells.[31] This observation may be one of the possibilities to explain the increased growth capacity seen in some of our patients.

We intend to follow-up this group of patients with repeated examinations, both with regard to their cytogenetic evolution and their colony growth pattern. The preliminary observation, in particular that mast cells grow from the bone marrow of patients both with systemic mastocytosis and urticaria pigmentosa, needs to be confirmed with other methods.

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Rearrangement of the T Cell Receptor γ-Chain Gene in Childhood Acute Lymphoblastic Leukemia

By Akio Tawa, Stephen H. Benedict, Junichi Hara, Nobumichi Hozumi, and Erwin W. Gelfand

We analyzed rearrangements of the T cell receptor γ-chain (T_γ) gene as well as rearrangements of the T cell receptor β-chain (T_β) gene and immunoglobulin heavy-chain (IgH) gene in 68 children with acute lymphoblastic leukemia (ALL). All 15 patients with T cell ALL showed rearrangements of both T_β and T_γ genes. Twenty-four of 53 non-T, non-B ALL patients (45%) showed T_γ gene rearrangements and only 14 of these also showed T_β gene rearrangements. Only a single patient rearranged the T_β gene in the absence of T_γ gene rearrangement. The rearrangement patterns of the T_γ gene in non-T, non-B ALL were quite different from those observed in T cell ALL, as 20 of 23 patients retained at least one germline band of the T_γ gene. In contrast, all T cell ALL patients showed no retention of germline bands. These data indicate that rearrangement of the T_γ gene is not specific for T cell ALL. Further, the results also suggest that T_γ gene rearrangement precedes T_β gene rearrangement. The combined analysis of rearrangement patterns of IgH, T_β, and T_γ genes provides new criteria for defining the cellular origin of leukemic cells and for further delineation of leukemia cell heterogeneity.

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HUMAN LYMPHOCYTES characteristically express distinct surface antigens and on this basis childhood acute lymphoblastic leukemia (ALL) has been subdivided into three major groups: T cell ALL, B cell ALL and non-T, non-B ALL based on the failure to express either T cell associated antigens, including the sheep erythrocyte receptor or surface immunoglobulin.[1] Reactivities with several antibodies associated with cells of B lineage suggested that most non-T, non-B ALL are B cell in origin; this has been confirmed by studying rearrangements of immunoglobulin genes.[2-6] Molecular cloning of the gene encoding the T cell receptor β-chain (T_β)[7] has more recently provided additional opportunities to study lineage commitment at the DNA level.[8-10]

On the basis of such studies, doubt has been cast on the lineage specificity of Ig heavy chain (IgH) gene rearrangements for B cells[11-13] and the specificity of T_β gene rearrangements in T cells.[10,14-16] Although exceptions to the rule are rare, there are nevertheless several descriptions of IgH gene rearrangement in T cell malignancies[11,13] and acute myelogenous leukemia.[12,17] Perhaps more striking was our finding that in 39 patients with non-T, non-B ALL who had rearranged IgH genes, ten had rearrangement of the T_β gene as well.[15]

Lymphoblastic leukemia is regarded as a monoclonal expansion of lymphocytes at specific stages of differentiation. The analyses of Ig and T_β gene rearrangements have offered new information about normal lymphocyte differentiation.[2,3,14,15,18,19] Recently, two groups isolated cDNA clones for the human T cell receptor γ-chain (T_γ) gene and demonstrated T_γ gene rearrangements in T cell lines and T cell malignancies.[20-22] Some data suggest that T_γ genes are rearranged and transcribed in the very early stage of T cell differentiation before T_β genes,[23-25] and T_γ transcripts have only been found in cytotoxic T cells.[24,26] Waldmann et al[10] reported that clonal T cells had rearranged T_γ genes, whereas the B cell populations, including those with T_β gene rearrangement, did not have rearrangement of this gene complex. To determine the lineage specificity of T_γ gene rearrangements, we analyzed leukemic cells from 15 patients with T cell ALL and 53 patients with non-T, non-B ALL derived from cells of B lineage. We examined the relationship between surface antigen expression and, IgH, T_β, and T_γ gene rearrangements, in order to determine whether the T_γ gene undergoes rearrangement before T_β gene rearrangement and to define specific rearrangement patterns in T cell and non-T, non-B ALL.

METHODS

Bone marrow cells. Bone marrow samples were obtained at the time of diagnosis and before initiation of treatment. Cell morphology was examined by Wright-Giemsa staining and cytochemistry. The percentage of leukemic cells in bone marrow samples was >85%. Mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation. Analysis of cell surface markers using a panel of monoclonal antibodies, sheep erythrocyte-rosette formation and surface and cytoplasmic immunoglobulin were carried out as described previously.[4,27,28]

DNA preparation and Southern blot analysis. High molecular weight DNA was extracted from the mononuclear cells of each patient. DNA was digested with the restriction endonucleases *Eco*RI or *Bam*HI. Digested DNA was size-fractionated by agarose-gel electrophoresis and transferred to nitrocellulose filters. The filters were hybridized to nick-translated or randomly primed ^{32}P-labeled probes.[29]

Gene probes. The human IgH gene probe was an *Eco*RI fragment that contained the constant region of μ ($C\mu$, 1.3 kb *Eco*RI fragment). The $C\mu$ probe identified a 19 kb *Bam*HI fragment[4] in germline DNA. A *Bgl*II-*Eco*RV fragment of the T_β cDNA clone YT-35 was used as a T_β constant region gene probe ($C\beta$, 0.8 kb *Bgl*II-*Eco*RV fragment). The $C\beta$ probe detected a germline 24 kb *Bam*HI fragment containing both β1 and β2 and 11 kb and 4 kb *Eco*RI fragments containing β1 and β2, respectively.[15] These probes

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have been described elsewhere.[4,15] The T_γ constant region probe was a *Pst*I-*Bam*HI genomic fragment (0.3 kb) containing DNA located 5′ to the *Bam*HI restriction site in exon 1 of the $C\gamma 1$ gene and hybridizing to both $C\gamma 1$ and $C\gamma 2$ (Fig 1). With *Eco*RI, the $C\gamma$ probe recognizes 5.4 kb ($C\gamma 1$) and 7 kb ($C\gamma 2$) germline bands. Using *Bam*HI, the $C\gamma$ probe recognizes 21 kb and 15 kb for $C\gamma 1$ and $C\gamma 2$, respectively. The $J\gamma$ probe was a *Hind*III-*Eco*RI genomic fragment (0.7 kb). The $J\gamma$ probe recognizes $J\gamma 1.3$ and $J\gamma 2.3$[30] fragments of 1.8 kb and 3.3 kb, respectively with *Eco*RI, and 21 kb and 15 kb, respectively, with *Bam*HI. The $C\gamma$ and the $J\gamma$ probes recognized identical *Bam*HI fragments.[20-22] $C\mu$ germline clones were kindly provided by Drs T.H. Rabbitts and G. Matthyssens,[31] YT-35 by Dr T.W. Mak,[7] the $C\gamma$ probe by Dr J.G. Seidman,[21] and the $J\gamma$ probe by Dr T.H. Rabbitts.[20]

There are at least five joining segment genes ($J\gamma 1.1$, $J\gamma 1.2$, $J\gamma 1.3$, $J\gamma 2.1$, and $J\gamma 2.3$) and two constant region genes ($C\gamma 1$ and $C\gamma 2$) in the human germline $T\gamma$ genome.[20-22] Following *Eco*RI digestion, the $C\gamma$ probe can recognize only the deletion of germline bands but can not detect a change of fragment size accompanying $T\gamma$ gene rearrangement, since as shown in Fig 1, there are *Eco*RI restriction sites between $J\gamma 1.3$ and $C\gamma 1$ as well as between $J\gamma 2.3$ and $C\gamma 2$.[21,22] For this reason, estimates of non-germline configuration were confirmed using the $J\gamma$ probe.

RESULTS

We analyzed surface markers and DNA from cells of 68 patients with ALL. Fifteen of 68 patients were diagnosed as having T cell ALL and the remaining 53 were classified as non-T, non-B ALL, based on studies using a panel of monoclonal antibodies, E-rosette formation, and staining for cytoplasmic and surface immunoglobulin. Among the 53 patients classified as non-T, non-B ALL, three groups could be distinguished: CALLA-negative–ALL, 6 patients; CALLA-positive–ALL, 35 patients; and cytoplasmic μ-chain–positive pre-B ALL, 12 patients.

T cell ALL. Thirteen of the 15 T cell ALL patients demonstrated the normal germline configuration of the IgH gene; two patients had rearrangements of one allele and a germline configuration of the other, as described previously.[32] Following *Bam*HI and *Eco*RI digestion, all 15 DNA samples had rearrangements of the T_β gene: nine patients had rearrangements of both alleles, and in six patients one allele was rearranged and the other deleted. Results of these studies are summarized in Table 1.

Figure 2 shows the patterns of T_γ gene rearrangements in these 15 patients. Using the $C\gamma$ probe, DNA samples from all T cell ALL patients were analyzed after *Eco*RI restriction endonuclease digestion. The normal germline configuration is shown in lane G (Fig 2A) as a 5.4 kb ($C\gamma 1$) band and a 7 kb ($C\gamma 2$) band. None of the T cell ALL patients retained germline configuration of both alleles. The majority of patients (12 of 15) demonstrated deletion of the 5.4 kb band and retention of the 7.0 kb band (lane 1). In three of 15 patients, the intensity of the 5.4 kb band was sufficiently lower than the 7.0 kb band (lane 2) to suggest deletion of one of the 5.4 kb bands. This was confirmed by densitometry of patient compared with control samples. Because the $C\gamma$ probe can recognize only deletions using *Eco*RI, 11 of the patients were reevaluated using the $J\gamma$ probe with *Eco*RI. Results with the $J\gamma$ probe were entirely consistent with the $C\gamma$ results. That is, samples that showed deletion of $\gamma 1$ with the $C\gamma$ probe showed no γ_1 germline with the $J\gamma$ probe and the number of rearranged bands identified by the $J\gamma$ probe was the same as seen with a *Bam*HI cut and the $C\gamma$ probe. Two (patients no. 11 and 15) of the three patients showing single allele deletion of γ_1 with the $C\gamma$ probe and *Eco*RI were re-examined with the $J\gamma$ probe. Both patients showed retention of germline band and a single rearranged band presumably γ_2.

Table 1. Surface Antigen Expression and Rearrangement Patterns of IgH, T_β, and T_γ Genes in 15 Patients With T Cell ALL

	Surface Markers*							T_γ Gene‡	
Patient No.	T11	6	4	8	3	IgH Gene†	T_β Gene†	*Eco*RI	*Bam*HI
1	–	–	–	–	+	R/G	R/R	2§	ND
2	20	–	+	30	–	R/G	R/R	1	2
3	+	30	29	+	+	G	R/R	1	3
4	+	18	–	20	18	G	R/R	1	2
5	+	–	42	45	33	G	R/R	1	2
6	+	+	+	+	30	G	R/R	1	2
7	+	17	29	34	+	G	R/R	1§	ND
8	+	–	–	–	+	G	R/R	1	2
9	+	+	40	40	+	G	R/D	1	ND
10	+	44	19	–	+	G	R/R	1	1
11	+	+	10	+	15	G	R/D	2	3
12	+	+	+	42	–	G	R/D	1	2
13	+	–	+	+	–	G	R/D	1	2
14	+	30	–	+	–	G	R/D	1	2
15	+	+	+	+	–	G	R/D	2	3

Abbreviation: ND, not determined.

*A minus sign denotes <10% positive cells and a plus sign >50% positive cells; numbers are specific percentages of positive cells.

†G denotes germline, R/G one allele rearranged and one allele germline, R/R two alleles rearranged, R/D one allele rearranged and one allele deleted.

‡Numbers denote rearrangement patterns observed with the $C\gamma$ probe, as represented in Fig 2, and not bands of the same size.

§These patients were not evaluated with the $J\gamma$ probe.

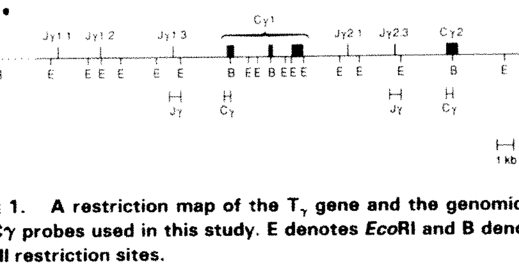

Fig 1. **A restriction map of the T_γ gene and the genomic $J\gamma$ and $C\gamma$ probes used in this study. E denotes *Eco*RI and B denotes *Bam*HI restriction sites.**

Following *Bam*HI digestion of normal cell DNA and hybridization with the $C\gamma$ probe, the germline configuration consists of two bands, 21 kb and 15 kb (Fig 2B, lane G). In each of 12 T cell ALL samples digested with *Bam*HI, one of three patterns demonstrated in Fig 2B was observed and the rearrangement patterns are summarized in Table 1. Lane 1 (Fig 2B) shows one rearranged band; one patient had this pattern. Eight patients had two rearranged bands (lane 2), and three patients (patients no. 3, 11, and 15) had three rearranged bands (lane 3). An *Eco*RI cut probed with the $J\gamma$ probe showed a singe rearranged $\gamma 2$ band in these patients. Analysis of the T_β locus also did not show three non-germline bands. The observation of three non-germline bands using *Bam*HI and the $C\gamma$ probe is therefore of interest. Extra

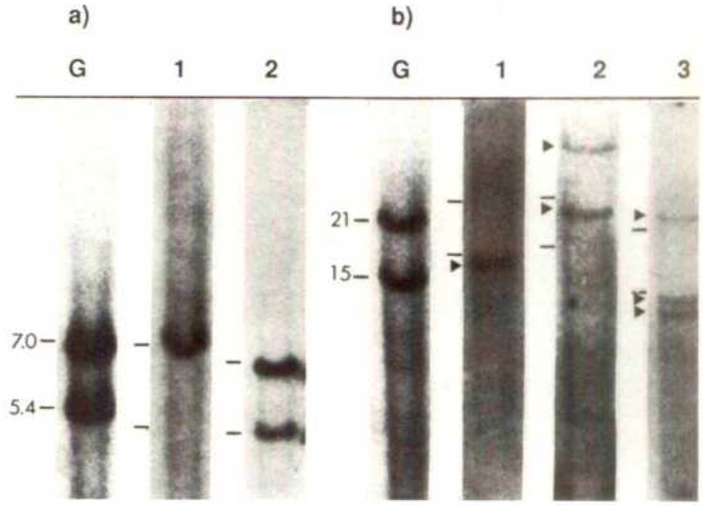

Fig 2. The rearrangement patterns of T_γ gene in T cell ALL patients (identified in Table 1). (A) *Eco*RI digestion. Lane G shows the germline control, lane 1 the sample from patient no. 4, and lane 2 the sample from patient no. 15. (B) *Bam*HI digestion. Lane G shows the germline control, lane 1 the sample from patient no. 10, lane 2 the sample from patient no. 4, and lane 3 the sample from patient no. 15. The germline positions obtained on the same gel with control DNA are indicated by lines and rearranged bands are indicated by arrows on the gels. The gels are not aligned because the studies were carried out at different times. Blots were probed with the Cγ probe.

bands of this nature usually suggest polymorphism of the DNA, clonal evolution of the leukemia, or that the leukemic cells had an abnormal karyotype. Karyotypic analysis was not available for these patients. Since *Eco*RI separates Jγ1.3 and Jγ2.3 from the other Jγ loci,[30] it is possible that Jγ1.1, Jγ1.2, Jγ2.1, or some as yet described Jγ locus was involved in the non-germline band detected by *Bam*HI.

Non-T, non-B ALL. All 53 DNA samples from patients in the non-T, non-B ALL group showed rearrangements of the IgH gene. Fifteen of 53 DNA samples showed rearrangement of the T_β gene following *Bam*HI and *Eco*RI digestion. We analyzed 39 patients' DNA for Tγ gene rearrangement after both *Eco*RI and *Bam*HI digestion, ten after only *Bam*HI digestion, and four after *Eco*RI digestion using the Cγ probe. The rearrangement patterns following *Eco*RI digestion are shown in Fig 3A. Of the 43 samples analyzed, 30 demonstrated germline configuration (Fig 3A, lane G). Three patients showed complete deletion of the 5.4 kb band (lane 1), whereas in eight patients a 5.4 kb band of decreased intensity relative to control was detected (lane 2) suggesting deletion of one of the 5.4 kb bands. In two patients a third pattern not observed in T-ALL was demonstrated consisting of a 7.0 kb band of decreased intensity compared with that of the 5.4 kb band (lane 3), suggesting deletion of one of the 7.0 kb Cγ2 bands. Such a deletion might suggest chromosomal abnormality.

Figure 3B shows the patterns of T_γ gene rearrangement after *Bam*HI digestion. Twenty-six of 49 samples analyzed retained the germline configuration (Fig 3B, lane G). In the remaining 23 patients, six different patterns of rearrangement were observed. Two patients had a single rearranged band (lane 1) and one patient showed two rearranged bands without germline bands (lane 2). Four patients manifested two rearranged bands with a 15 kb Cγ2 germline band (lane 3). As illustrated for the patients in lanes 1 through 3, the rearranged band in each case was ∼18 kb. Five patients demonstrated one rearranged band accompanied by both 21 kb and 15 kb germline bands (lane 4). Ten patients had one

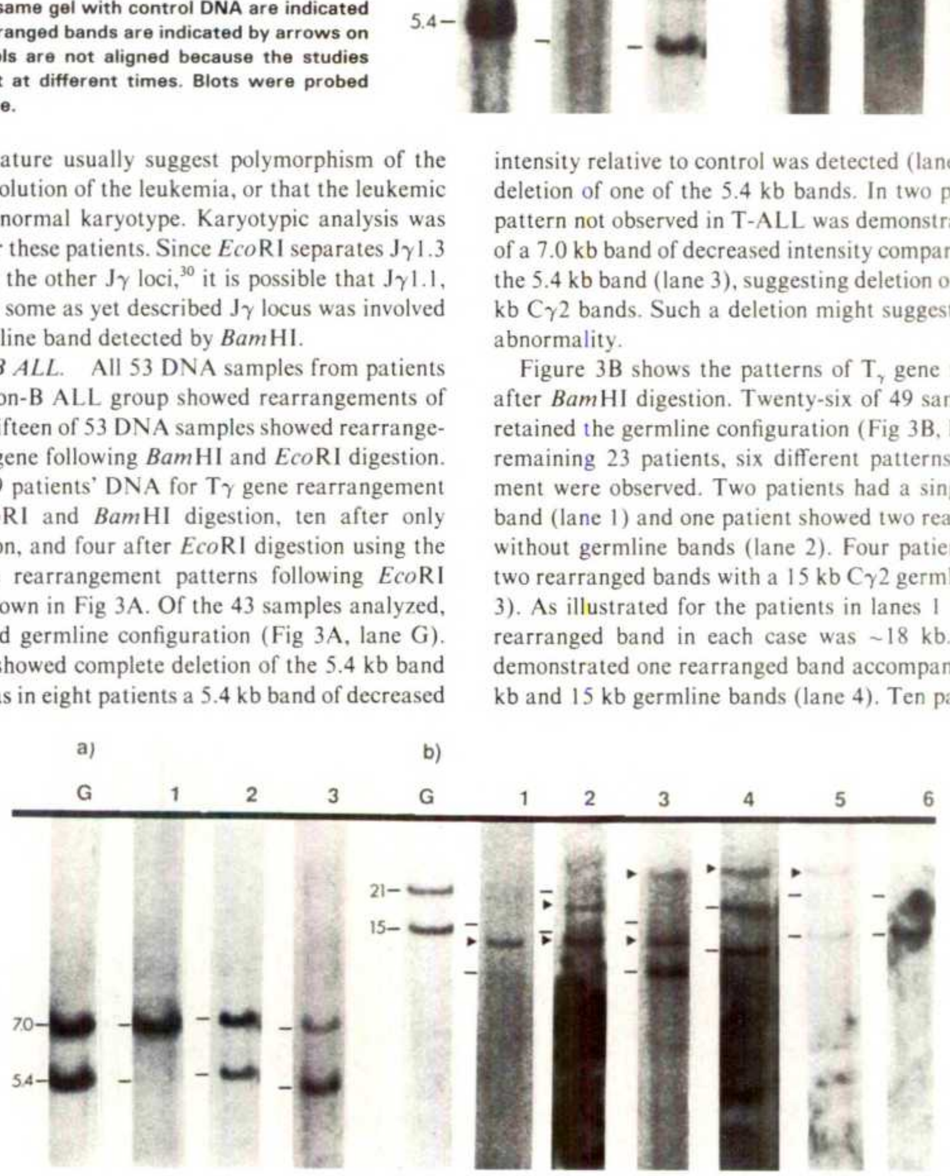

Fig 3. The rearrangement patterns of T_γ gene in non-T, non-B ALL patients (identified in Table 2). (A) *Eco*RI digestion. Lane G shows germline configuration and represents the sample from patient no. 35, lane 1 from patient no. 26, lane 2 from patient no. 38, and lane 3 from patient no. 33. (B) *Bam*HI digestion. Lane G shows germline configuration and represents the sample from patient no. 40 (not shown in Table 2), lane 1 from patient no. 25, lane 2 from patient no. 27, lane 3 from patient no. 20, lane 4 from patient no. 35, lane 5 from patient no. 19, and lane 6 from patient no. 18. The germline positions obtained on the same gel with control DNA are indicated by lines and rearranged bands are indicated by arrows on the gels. The gels are not aligned because the studies were carried out at different times. Blots were probed with the Cγ probe.

rearranged and one germline band (lane 5). Only one patient had the pattern shown in lane 6, namely, one 15 kb Cγ2 germline band with deletion of the 21 kb Cγ1 band. DNA samples from 27 patients were hybridized with the Jγ probe following *Bam*HI digestion. Identical results were obtained.

Overall (Table 2), we observed T_γ gene rearrangement in 24 of 53 (45%) non-T, non-B ALL patients. Twelve (of 39) samples showed non-germline configuration of the T_γ gene after both *Eco*RI and *Bam*HI digestion. In eight patients who manifested a rearranged band following *Bam*HI digestion, there was a germline configuration following *Eco*RI digestion (indicating rearrangement in Cγ1). One patient (of four) analyzed following only *Eco*RI digestion and two (of ten) patients following only *Bam*HI digestion showed T_γ genes in a non-germline configuration. Following *Bam*HI digestion, the germline bands identified by the Cγ probe are quite large and this size could mask small rearrangements. For this reason, *Eco*RI cuts of 27 cases were probed with the Jγ probe.[30] No additional rearrangements were found. Twelve patients that were germline using the Cγ probe for *Eco*RI and *Bam*HI cuts were re-examined with the Jγ probe and found to be germline (not shown). Fifteen patients (designated in Table 2) who were found to be rearranged with *Bam*HI and/or deleted using *Eco*RI and the Cγ probe were re-examined with the Jγ probe. In 13 of these cases, the same number of rearranged bands seen with *Bam*HI and the Cγ probe was observed with *Eco*RI and the Jγ probe thus confirming the results in Table 2. Figure 4 presents representative results of these studies. Using *Eco*RI and Cγ probe, patient 27 was found (Table 2) to have deleted both γ_1 alleles and using *Bam*HI, two rearranged bands were observed, presumably γ_2. In Fig 4, lane 1, these results were confirmed using *Eco*RI and the Jγ probe; two rearranged bands were observed. Faint germline bands were contributed by contaminating nonleukemic cells. Patient no. 36 was found (Fig 3, Table 2) to have deleted a single γ_1 allele using the Cγ probe and *Eco*RI, and to have two rearranged bands (presumably one γ_1 and one γ_2) using *Bam*HI. In lane 2 of Fig 4, patient no. 36 had two rearranged bands and a γ_2 band retained, the same as with *Bam*HI. Patient no. 39 was not previously examined with the Cγ probe and *Eco*RI. However, using the Cγ probe and *Bam*HI we observed γ_1 and γ_2 in germline and one rearranged band (Table 2). These results were confirmed (Fig 4, lane 3) using the Jγ probe and *Eco*RI. In two patients (patients no. 21 and 35) with *Bam*HI rearrangements, no rearrangement was seen using the Jγ or Cγ to probe an *Eco*RI cut. This suggests usage of Jγ1.1, Jγ1.2,[31] or unknown Jγ1 loci that may be located upstream of Jγ1.3.[30]

Of the 24 patients with T_γ gene rearrangement, 14 (26%) also showed rearrangements of the T_β gene. Among the 29 patients who showed a germline configuration of the T_γ gene, 28 were also in germline for the T_β gene. There was one

Table 2. The Rearrangement Patterns of IgH, T_β, and T_γ Genes in 24 Non-T, Non-B ALL Patients

			T_γ Gene†	
Patient No.*	IgH Gene	T_β Gene	*Eco*RI	*Bam*HI
16‡	R/R	G	G	5
17	R/G	R/R	G	5
18	R/R	R/D	G	6
19‡	R/R	G	2	5
20	R/D	R/R	G	3
21‡	R/G	G	G	4
22‡	R/R	R/G	G	5
23‡	R/D	R/G	G	5
24	R/R	G	G	5
25	R/D	R/G	1	1
26‡	R/G	R/G	1	1
27‡	R/R	G	1	2
28‡	R/D	R/D	2	3
29‡	R/G	R/R	2	3
30‡	D/D	R/D	2	5
31	R/G	R/R	2	ND
32	R/D	G	3	4
33	R/G	R/R/G	3	5
34‡	R/R	G	ND	4
35‡	R/R	G	G	4
36‡	R/G	R/G	2	3
37	R/R	G	2	5
38‡	R/D	R/G	2	5
39‡	R/D	G	ND	4

*Patients no. 16-19, CALLA-negative ALL; patients no. 20-34, CALLA-positive ALL; patients no. 35-39, pre-B ALL.

†Numbers denote specific patterns observed with the Cγ probe, as represented in Fig 3, and not sizes of bands.

‡Patients that were also evaluated using the Jγ probe.

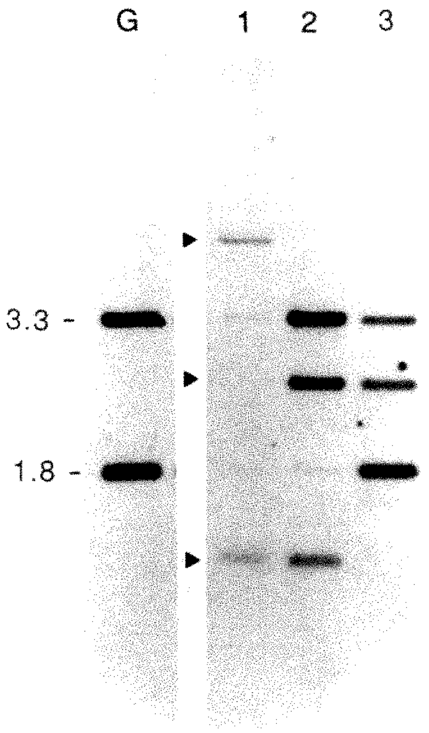

Fig 4. Rearrangement patterns of the T_γ gene examined with the Jγ probe in non-T, non-B ALL. DNA was digested with *Eco*RI and blots probed with the Jγ probe. Lane G shows the germline bands of 3.3 Kb (γ_2) and 1.8 Kb (γ_1). Representative patients presented were patient no. 27 (lane 1), patient no. 36 (lane 2), and patient no. 39 (lane 3).

patient, a single exception, who demonstrated a rearrangement of one allele and deletion of the other allele of the T_β gene, but with retention of the germline configuration of the T_γ gene after both *Eco*RI and *Bam*HI digestion. The patterns of T_β and T_γ gene rearrangement in this patient were identical when DNA was studied both at the time of diagnosis and several months later at the time of relapse.

DISCUSSION

We analyzed leukemic cell DNA from 68 children with lymphoblastic leukemia for IgH, T_β, and T_γ gene rearrangement. All 15 patients with T cell ALL showed rearrangements of both the T_γ and T_β genes. Thus, as reported previously for T_β gene rearrangements in T cell ALL,[8-10] T_γ gene rearrangements are also characteristic for T lineage cells. The patterns of rearrangement did not correlate with any particular pattern of T cell antigen expression. However, just as rearrangements of the T_β gene are not restricted to cells of T lineage,[10,14-16] we now show that rearrangement of the T_γ gene is similarly not restricted to cells of T lineage.[33] In the present study, 38% of the 68 patients examined with ALL had both IgH and T_γ gene rearrangement and 24% had both IgH and T_β gene rearrangements.

Three separate studies have shown that 8% to 11% of lymphoid malignancies demonstrate both IgH and T_β gene rearrangement.[10,14-16] This combination of immunoglobulin and T cell receptor gene rearrangement may be a result of neoplastic transformation or may occur during normal lymphocyte differentiation. Several studies reporting IgH gene rearrangement and expression in mouse thymocytes and lymphocytes[34,35] suggest that this combined gene rearrangement may occur in normal cells. In this case, the combined gene rearrangement may reflect common molecular mechanisms leading to immunoglobulin and T cell receptor gene rearrangement. This concept is consistent with the observation that immunoglobulin heavy and light chain gene segments and T cell receptor β gene and γ gene segments have similar recombination recognition sequences,[36-42] and with the evidence[43] indicating that both B and T cells use a common recombinase system in the mouse. If this concept applies to human lymphoid tumors, a common recombinase may lead to rearrangement of both immunoglobulin and T cell receptor genes in lymphoid malignancies.

There are two constant region genes (Cγ1 and Cγ2) in the human germline T cell receptor γ-chain genome.[20-21] Based on recent studies,[20-22,42] constant region probes recognize 5.4 kb and 7.0 kb *Eco*RI germline fragments containing Cγ1 and Cγ2, respectively and also identify 21 kb and 15 kb *Bam*HI germline fragments containing Cγ1 and Cγ2, respectively. In 15 T cell ALL patients, 12 had no 5.4 kb *Eco*RI fragment containing Cγ1, and the other three probably had only one Cγ1 allele, judged by the intensity of the 5.4 kb band. Moreover, all T cell ALL samples digested with *Bam*HI did not contain any of the two germline fragments containing Cγ1 and Cγ2. These results suggest that in the majority of T cell ALL, T_γ genes have undergone rearrangement to the γ^2 gene of both alleles. Quertermous et al reported that most T cell malignancies showed biallelic Cγ1 deletion with *Eco*RI digestion and had no germline band hybridized to a J region probe with *Eco*RI digestion.[22] Our results are consistent with these findings.

Surprisingly, 45% of non-T, non-B ALL patients rearranged the T_γ gene and similar to our previous study, 26% rearranged the T_β gene.[15] Among the 24 non-T, non-B ALL patients with T_γ gene rearrangement, only three (patients no. 25, 26, and 27) showed the same rearrangement patterns as those observed in T cell ALL. The other patients retained a 15 kb germline band containing Cγ2 with *Bam*HI digestion and five patients (patients no. 21, 32, 34, 35, and 39) had two germline bands indicating at least one allele in germline configuration. The intensity of the germline band argued against the possibility of its being due to contamination by normal cells. In addition, 17 of 20 patients had no residual germline bands of IgH or T_β genes. Our data and those of others[22] suggest that biallelic γ^2 rearrangement is characteristic for T cell ALL and retention of germline γ^2 is the rule in non-T, non-B ALL.

Most B lineage malignancies have both IgH gene alleles rearranged[2,6] and almost all T lineage cell malignancies show biallelic T_β gene rearrangement.[9,10,16] In contrast, B lineage malignancies with rearrangement of both alleles of the T_β gene, or T lineage cell malignancies with rearrangement of both alleles of the IgH gene are rare; in most cases, there is one residual germline allele of the IgH or T_β gene.[11,13,15,16,32] The rearrangement patterns for these three genes in our 68 patients are summarized in Table 3. These data indicate the difficulty in assigning lineage based solely on the rearrangement pattern of a single gene. As illustrated in Table 3, the further examination of three rearranging genes indicate the complex heterogeneity of childhood ALL.

Some reports have suggested that the rearrangement and transcription of T_γ, T_β, and T cell receptor α chain genes during T cell ontogeny is sequential.[23-25,44-46] To determine whether T_γ gene rearrangement precedes T_β gene rearrangement, we analyzed the relationship between T_γ and T_β gene rearrangement in 68 ALL patients. All 15 T cell ALL patients and 14 of 53 non-T, non-B ALL patients showed rearrangements of both T_γ and T_β genes, and 28 of 53 non-T,

Table 3. Rearrangement Patterns of IgH Gene, T_β Gene, and T_γ Gene in 68 Patients with ALL

		T Cell ALL	Non-T, Non-B ALL
IgH Gene	G*	13	0
	G/R	2	12
	R	0	41
T_β Gene	G	0	38
	G/R	0	7
	R	15	8
T_γ Gene†	G	0	29
	G/R	0	20
	R	14	3

*G denotes germline configuration, G/R, rearrangement with a germline allele, and R, rearrangement without a germline allele.

†Patients no. 1 and 31 were not included, since T_γ gene rearrangement was not analyzed following *Bam*HI digestion.

non-B ALL patients showed germline configuration of both genes. Ten non-T, non-B ALL patients with germline configuration of the T_β gene showed rearrangements of the T_γ gene, and there was only one patient who showed germline configuration of the T_γ gene and rearrangement of the T_β gene. It is possible that non-T, non-B leukemic cells are not appropriate for drawing conclusions about T cell gene rearrangements in normal cells. Nevertheless, these results are similar to the observations of others[23-25] and are consistent with the hypothesis that the T_γ gene undergoes rearrangement before rearrangement of the T_β gene.

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In Vitro Interaction of Recombinant Tumor Necrosis Factorα and All-Trans-Retinoic Acid With Normal and Leukemic Hematopoietic Cells

By Andreas Tobler, Reinhold Munker, Daniel Heitjan, and H. Phillip Koeffler

Both human recombinant tumor necrosis factor alpha (TNFα) and all-*trans*-retinoic acid (RA) inhibit the in vitro clonal growth of human myeloid leukemic cells. We investigated the in vitro interaction of TNFα and RA with normal and a variety of leukemic myeloid cells. With the promyelocytic HL-60 cells, TNFα ($\geq$2.5 U/mL) in combination with RA synergistically inhibited clonal growth; TNFα at lower concentrations ($\leq$1 U/mL) plus RA (10^{-9} mol/L) were antagonistic in their inhibition of growth. The ability of RA (10^{-8} mol/L) plus TNFα (2.5, 5 U/mL) to enhance differentiation of HL-60 cells paralleled their ability to inhibit clonal growth of these cells. In addition, RA (10^{-9} to 10^{-7} mol/L) increased the number of TNFα receptors on HL-60 cells 1.3- to 1.7-fold without changing the affinity for the TNFα receptor. With the more immature KG-1 myeloblasts, concentrations of TNFα >10 U/mL synergistically interacted with RA to inhibit clonal growth; at lower concentrations of TNFα (<10 U/mL), RA appeared to inhibit the expected effect of TNFα. KG-1 cells were not induced to differentiate with either agent alone or in combination. With four of nine leukemic patients, TNFα in combination with RA (10^{-7} mol/L) inhibited leukemic clonal growth to a greater extent than each agent alone. No marked effect of the combined treatment was seen in two other patients. The RA reversed the inhibitory action of TNFα on normal human granulocyte-macrophage colony forming cells (GM-CFC) and on clonal growth of leukemic cells from three patients. Our study suggests that TNFα and RA interact in a complex manner with normal and leukemic hematopoietic cells.

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TUMOR NECROSIS factor alpha (TNFα) is a protein produced by myeloid cells. The TNFα was originally identified in the serum of endotoxin treated mice who were previously sensitized with bacillus Calmette-Guerin.[1] Human and murine TNFα recently have been purified, and the genes coding for these cytokines have been cloned and expressed.[2-4] Initial in vitro studies suggested that TNFα might be preferentially cytotoxic to certain malignant cells as compared with their normal counterpart (for a review, see ref. 5). We and others recently demonstrated that low concentrations of human recombinant TNFα effectively suppressed clonal growth in soft agar of cells from a variety of human myeloid leukemic cell lines and leukemic cells from patients.[6-8] In contrast, in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) clonal growth of normal human myeloid progenitor cells (GM-CFC) was inhibited only at much higher concentrations.[6,7,9] Studies further revealed that TNFα induces HL-60 to differentiate towards monocytes/macrophages.[6,7,10-12]

Retinoids in vitro effectively inhibited clonal growth and induced differentiation of myeloid leukemic cells harvested from patients and cell lines.[13-17] By contrast, retinoids stimulated clonal growth of normal human myeloid progenitor cells.[14,18] A logical extension of these studies was to investigate the combined in vitro effect of all-*trans*-retinoic acid (RA) and TNFα on clonal proliferation and differentiation of both human myeloid leukemic cells and normal human GM-CFC.

MATERIALS AND METHODS

Chemicals. The RA was a generous gift from M. Sherman (Hoffmann-La Roche, Inc, Nutley, NJ). Stock solution of 2.5 × 10^{-3} mol/L RA was prepared in 100% ethanol and stored at −20°C under a nitrogen atmosphere for no longer than 14 days. Before use, dilutions were made in phosphate-buffered saline (PBS) to the final concentrations of 1 × 10^{-7} to 1 × 10^{-9} mol/L. The concentrations of ethanol (<0.04%) had no influence on colony formation in control plates as compared with culture plates not containing ethanol. All experiments were performed in subdued light. The TNFα was generously provided by Dr M. Shepard (Genentech Inc, San Francisco). The stock solution (1 mg corresponds to 3.7 × 10^7 U; 1 U corresponds to 27 pg) was kept at 4°C in a sealed container. Before use, TNFα was diluted in alpha modified minimum essential medium (Flow Laboratories, Inc, Rockville, MD) plus 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA) to final concentrations of 10,000 to 0.5 U/mL.

Cell preparations. The human myeloid cell lines HL-60 (passage 35, mean doubling time 32 hours)[19] and KG-1 (passage 30, mean doubling time 48 hours)[20] were maintained in culture T flasks (Miles Lab. Naperville, IL) containing alpha medium and 10% FCS. The cells were incubated in a humidified atmosphere with 7% CO_2. The medium was changed twice a week. All experiments were performed in the logarithmic growth phase of the cells. Normal human bone marrow cells were obtained from informed, healthy volunteers. Likewise, peripheral blood or bone marrow samples from leukemia patients were obtained after informed consent. The bone marrow or peripheral blood cells were layered over Ficol-Hypaque (density 1.077 g/mL) (Sigma Chemical Co, St Louis) and centrifuged for 40 minutes × 450 g. The light density, mononuclear cell fraction was washed twice in PBS and resuspended in alpha medium plus 10% FCS. Leukemia cells from all patients with chronic myelogenous leukemia (CML) contained the Philadelphia chromosome.

Colony formation assay by two layer agar technique. The colony formation assay was performed as previously described.[13,14] Briefly, an underlayer was plated in 1-mL portions in 35-mm petri

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dishes (Miles) containing 0.5% agar, alpha medium, 16% FCS and the various additives (TNFα, RA), and CSF. As a source of CSF, conditioned medium (CM) obtained from a human T lymphocyte cell line (Mo) (Mo-CM) was used at concentrations of 1.5% to 2.5%.[21] The overlayer (1-mL) contained cells, alpha medium, 16% FCS, antibiotics and 0.3% agar. The HL-60 promyelocytic leukemia cells form colonies without CSF. The KG-1 myeloblastic leukemia cells (early passage), normal human bone marrow and leukemic cells from patients required CSF for clonal growth. The culture dishes were incubated at 37°C in a humidified atmosphere with 7% CO_2 for ten to 13 days. Colonies (>40 cells) were counted using an inverted microscope.

Cytochemistry. For nitroblue tetrazolium reduction (NBT), a total of 1×10^6 cells/mL were incubated in an equal volume of 1.25 mg NBT (Sigma), 17 mg bovine albumin (Sigma), and 10^{-7} mol/L 12-*O*-tetradecanoylphorbol 13-acetate (Consolidated Midland, New Rochelle, NY) in alpha medium plus 10% FCS for 30 minutes at 37°C. Cytospin slides of the cells were stained with safranin (Difco Laboratories, Inc, Detroit) and the number of cells containing reduced blue black formazan deposits was counted.[14] For α-naphtyl acetate esterase (ANAE) the cells were stained according to routine hematologic methods,[22] kindly performed by Dr F. Naeim (Department of Pathology, UCLA). Morphology was assessed by Giemsa staining. In each of the various experiments at least 200 cells were examined. Cell viability was >90% in all experimental procedures, as determined by trypan blue exclusion.

Monoclonal antibodies, indirect immunofluorescence. The monoclonal antibodies anti–leu-M3 and Y2FITC (mouse IgG2) were obtained from Becton-Dickinson (Mountain View, CA). Anti–leu-M3 reacts with 70% to 90% of normal human peripheral blood monocytes and has marginal reactivity with peripheral blood granulocytes. Y2FITC was used as isotypic control to define background staining. Analyses were performed on EPICS C flow cytometer (Coulter Electronics, Hialeah, FL), as described.[24]

Binding of ^{125}I-TNFα by HL-60 cells. The TNFα was iodinated by the Iodogen method as described.[24] Briefly, 10 μg of purified recombinant protein was incubated for ten minutes at 4°C in an Iodogen (Pierce, Rockford, IL) coated glass tube with 1 mCi of ^{125}I-TNFα (Amersham Corp, Arlington Heights, IL). Free iodide was separated from TNFα on a Sephadex G-25 column. The HL-60 cells were incubated with ^{125}I-TNFα (10^4 to 9×10^5 cpm) at 23°C for 90 minutes in alpha-medium, 10% FCS, and 20 mol/L Hepes (Sigma), washed extensively and the cell pellets counted in a Beckman gamma-counter. Previous experiments by us showed that at 23°C the peak of specific binding was reached at 45 minutes.[25] Nonspecific binding was determined by incubating the cells with a 100-fold excess of unlabeled TNFα. The specific activity of ^{125}I-TNFα was determined with a self-displacement experiment in which KG-1 cells were incubated with 0.4 to 10 μg unlabeled TNFα in the presence of $4 \times 10^4 - 5 \times 10^5$ cpm ^{125}I-TNFα and found to be 4.2×10^5 cpm/μg. Scatchard curves, the receptor number per cell and the affinity constants of binding were calculated with a computer program as described.[25] Under reducing conditions, iodinated recombinant TNFα gave a single band of the approximate mol wt of 17,000 on a 12% SDS-PAGE gel.[25] Under nonreducing conditions and longer exposure an additional faint band at ~34,000 daltons was observed (<2% of the intensity of the 17,000 band), indicating that little TNFα aggregates in vitro. Further, iodinated TNFα retained for over 2 months its biologic activity as measured by the inhibition of colony formation of HL-60 cells.

Statistical methods. Dose response curves were fit for each target (HL-60, KG-1 cells) and for each anti-tumor drug (RA, TNFα) by weighted least squares logistic regression.[26,27] At each percent inhibition, we computed a combination index (CI) for the additive (mutually exclusive binding) model using the method of Chou and Talalay.[27] We computed a standard error for CI (SE[CI]) using the delta method.[28] Drug combinations resulting in a given percent inhibition were said to be synergistic if CI + SE(CI) < 1, antagonistic if CI-SE(CI) > 1, and additive if CI + SE (CI) = 1. Cooperation between the drugs was also assessed by computing the fractional inhibitory concentration and its SE for each test concentration. These computations were based on the isobol method as described.[29] The effect of adding RA to TNFα compared with TNFα alone in the leukemic patients was assessed by fitting a model linear in the logs of the data. Significance was determined by comparing the effects to its standard error, which results in normal *z* statistics.

RESULTS

We examined the effect of the combination of TNFα (0.5 to 100 U/mL) and RA (10^{-8}, 10^{-9} mol/L) on clonal growth of the HL-60 promyelocytic cell line (Fig 1A). The TNFα and RA showed a dose-dependent interaction. Statistical analyses of the data (see Materials and Methods) showed that concentrations of TNFα ≥ 2.5 U/mL in combination with RA (10^{-8}, 10^{-9} mol/L) synergistically inhibited clonal growth of HL-60 cells. Evidence for an antagonistic interaction of TNFα and RA was observed at TNFα concentrations up to 1 U/mL when combined with 10^{-9} mol/L RA. Similar interpretations of the results were obtained when the isobol method of analysis was applied.

In an attempt to determine possible mechanisms of this enhanced inhibitory effect of TNFα and RA in HL-60 cells, several experiments were performed. The ability of TNFα plus RA to induce differentiation of HL-60 to mature cells was studied by examining the capability of these cells to reduce NBT (Fig 1B). At the concentrations tested (5 and 2.5 U/mL for six days), TNFα alone induced a mean 24% ±

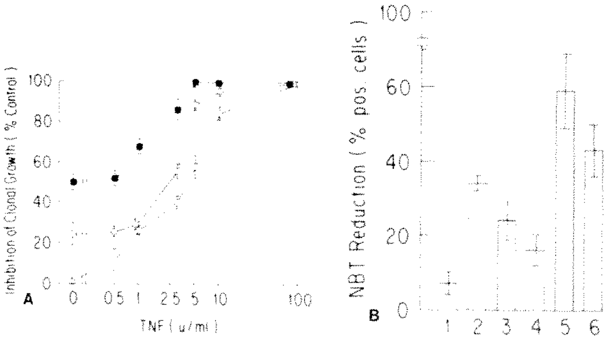

Fig 1. **(A) Dose response curves of inhibition of clonal growth of HL-60 cells by TNFα and RA. The cells were incubated with different concentrations of TNFα without (○) or with RA 10^{-8} mol/L (■) or 10^{-9} mol/L (□). Results are expressed as a mean percentage (±SD) of clonal growth inhibition in culture dishes containing the additives as compared with the control plates without additives. Three independent experiments were performed with triplicate dishes per point. Control plates contained a mean of 226 to 353 colonies per 1.5×10^3 plated cells. (B) Effect of TNFα and RA on induction of differentiation of HL-60 cells as measured by NBT reduction. Cells were incubated six days with different concentrations of TNFα and/or RA, harvested, and measured for NBT reduction. Data represent the means ± SD of three independent experiments done in duplicate flasks. 1, control (no TNFα, no RA); 2, RA (10^{8} mol/L); 3, TNFα (5 U/mL); 4, TNFα (2.5 U/mL); 5, TNFα (5 U/mL) plus RA (10^{-8} mol/L); 6, TNFα (2.5 U/mL) plus RA (10^{-8} mol/L).**

6% (±SD) and 16% ± 4% (±SD) of the HL-60 cells to reduce NBT, respectively. The RA alone (10^{-8} mol/L) induced differentiation of a mean 34% ± 3% (±SD) of HL-60 cells. Exposure of HL-60 cells for six days to TNFα (5 U/mL) plus RA (10^{-8} mol/L) significantly ($P < .001$) increased the number of these cells that reduced NBT (mean 59% ± 8% [±SD]) compared with the effect of each agent alone (mean 24% ± 6% [±SD] and 34% ± 3% [±SD] for TNFα and RA, respectively). Absolute numbers of HL-60 cells of one representative experiment were ($\times 10^6$/mL): control, 1.2; TNFα 5 U/mL, 0.6; TNFα 2.5 U/mL, 0.85; RA (10^{-8} mol/L), 0.75; TNF 5U/mL plus RA, 0.4; TNF 2.5 U/mL plus RA, 0.5. Lower concentrations of TNFα (0.5, 1.0 U/mL) in combination with RA (10^{-8} mol/L) showed no increase in the number of NBT positive cells, as compared with cells treated with RA alone; and TNFα alone showed no effect on NBT reduction at these concentrations (data not shown).

To determine the pathway of differentiation, HL-60 cells were exposed to TNFα plus RA (5 U/mL and 10^{-8} mol/L × 6 days, respectively) or to each agent alone (Table 1). Morphologically, 17% of the cells treated with RA alone showed granulocytic like differentiation; TNFα in combination with RA increased the number of differentiated cells to 62% with 20% of the cells showing morphological features of granulocytic-like cells and 42% having metamyelocytic- or monocytic-like appearance. In the presence of TNFα alone, 12% of the cells showed monocytic-like differentiation. No adherence was observed in cells treated with either TNFα alone or TNFα plus RA. Cytochemical analyses revealed that exposure to TNFα plus RA increased the number of ANAE positive cells to 35%, as compared with 15% ANAE positive cells when treated with TNFα alone, respectively. Fifteen percent of these cells (cultured with TNFα plus RA) were morphologically differentiated but were ANAE negative. Cytofluometric analyses showed that the combination of RA and TNFα increased the percentage of HL-60 cells expressing leu M3 (antigen expressed on monocytes but not granulocytes) to 24%, as compared with 15% and ≤2% in cells treated with TNFα or RA, respectively. Together, these results suggest that HL-60 cells when cultured with RA plus TNFα differentiate towards a mixed population of monocytic- and granulocytic-like cells.

Table 2. Binding Characteristics of ^{125}I-TNF α on HL-60 Cultured With RA

RA-dose	No. of Experiments	Receptors/Cell (Mean ± SD)	K_D of TNF Receptors (pmol/L)
0	n = 3	776 ± 39	42 ± 6
10^{-9}mol/L	n = 1	999	46
10^{-8}mol/L	n = 2	1201 ± 208*	64 ± 15
10^{-7}mol/L	n = 1	1338	54

HL-60 cells were cultured for five days in the presence of various concentrations of RA and then assessed for specific TNFα binding as described in Materials and Methods.

*$P \leq .05$ (determined by Student's t test).

The effect of RA on the expression of the TNFα receptors by HL-60 cells was examined as measured by the binding of ^{125}I-labeled recombinant human TNFα (Table 2 and Fig 2, for a representative Scatchard plot). The HL-60 cells were incubated for five days with several concentrations of RA (10^{-9} to 10^{-7} mol/L) and number of TNFα receptors per cell was determined. The wild type HL-60 cells expressed a single class of high affinity receptors (mean 776 ± 40 [±SD] receptors with a K_D binding affinity of 42 pmol/L). The RA increased expression of the number of TNFα receptors without a marked change in the affinity constants of binding of TNFα (1.3-, 1.6-, and 1.7-fold increase for cells cultured with 10^{-9} mol/L, 10^{-8} mol/L, and 10^{-7} mol/L RA, respectively as compared with wild-type HL-60 cells).

The effect of clonal growth of the combination of TNFα (1 to 1,000 U/mL) and RA (10^{-8} mol/L) was further studied using cells of the human myeloblastic line, KG-1, which are more immature than HL-60 cells and do not undergo differentiation in the presence of RA[14] (Fig 3). Clonal growth of KG-1 cells in early passages is dependent on CSF.[20] The KG-1 cells were less sensitive than HL-60 cells to the inhibitory action of TNFα (50% clonal inhibition [ED_{50}] at 15 U/mL for KG-1 cells as compared with 3.5 U/mL for HL-60 cells). By contrast, KG-1 cells were more sensitive than HL-60 cells to inhibition of growth by RA (ED_{50} 4×10^{-9} mol/L and 1×10^{-8} mol/L, respectively).[14] The combination of TNFα and RA resulted in various effects on clonal growth of KG-1 cells. The TNFα showed synergism with RA (10^{-8} mol/L) in the inhibition of clonal growth only at concentrations ≥10 U/mL. At lower concentrations of TNFα (<10 U/mL), the two agents were antagonistic in their inhibition of clonal growth of KG-1 cells. The TNFα

Table 1. Differentiation of HL-60 Cultured With TNFα and RA

Treatment (6 d)					
RA (10^{-8} mol/L)	TNFα (5 U/mL)	No. of Cells ($\times 10^6$/mL)	Morphology (Percent Differentiated Cells)	ANAE (Percent Positive Cells)	Leu-M3 (Percent Positive Cells)
–	–	1.4	≤1	≤1	≤2
+	–	0.9	17*	≤1	≤2
–	+	0.9	12†	15	15
+	+	0.7	62‡	35	24

HL-60 cells were cultured for six days with TNFα and/or RA and analyzed as described in Materials and Methods. Similar results were obtained in three independent experiments.

Abbreviations: ANAE, α-naphthylacetate esterase; RA, retinoic acid; TNFα, recombinant tumor necrosis factor α.

*Granulocytic-like cells.

†Monocytic-like cells.

‡Twenty percent granulocytic-like cells; 42% with metamyelocytic or monocytic features.

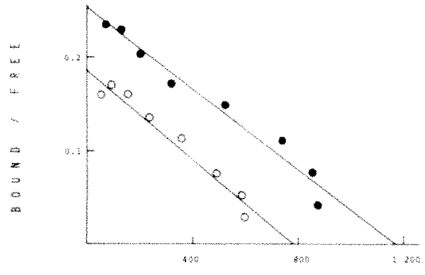

Fig 2. Scatchard plot of specific TNFα binding to HL-60 cells. HL-60 cells were cultured for five days either in the presence of RA (10^{-8} mol/L (●) or in the absence of RA (control) (○). 0.8 × 10^6 cells were then incubated with 10,000 to 900,000 cpm of ^{125}I-TNFα for 90 minutes at 23°C. Correlation coefficients were R = 0.986 for the control samples, and R = 0.980 for the RA-treated samples.

(2.5, 5.0 U/mL) and RA (10^{-8} mol/L) alone or in combination were not able to induce differentiation of KG-1 cells as determined by morphology and NBT-reduction (data not shown).

Diverse responses on clonal growth of leukemic cells freshly obtained from patients were observed when combinations of TNFα (1 to 10,000 U/mL) and RA (10^{-7} mol/L) were tested (Fig 4). The diagnoses are listed in the legend of Fig 4. In six of nine patients (no. 1 through 6) both RA and TNFα alone inhibited clonal growth to various degrees. With patients no. 1 through 4, the TNFα in combination with RA showed on average a greater inhibition of leukemic clonal growth ($P \leq .05$) as compared with TNFα alone. In two patients (no. 5 and 6), RA appeared to reduce the effect of TNFα at some but not all TNFα concentrations. In three patients (no. 7 through 9) TNFα alone inhibited leukemic growth, whereas RA alone stimulated leukemic growth; and when combined with TNFα, RA markedly decreased ($P \leq .05$) the inhibitory action of TNFα.

Further studies investigated the effect of the combination of TNFα and RA on GM-CFC (Fig 5). Normal human myeloid progenitor cells were ~2,800 times less sensitive to the inhibitory action of TNFα than HL-60 cells (ED_{50} at 10,000 U/mL and 3.5 U/mL TNFα, respectively) when CM from a human T lymphocyte cell line (Mo-CM) was used as a source of CSF. The RA alone (10^{-7} mol/L) enhanced GM-CFC formation ~1.6-fold. The addition of RA (10^{-7} mol/L) to the culture dishes containing TNFα (1 to 10,000 U/mL) either partially or completely reversed the inhibitory effect of TNFα on formation of GM-CFC. For example, at a concentration of 1,000 U/mL TNFα, a mean 40% ± 6% (±SD) of the GM-CFC were inhibited as compared with control plates; with the addition of 10^{-7} mol/L RA to plates containing 1,000 U/mL TNFα, GM-CFC formation was

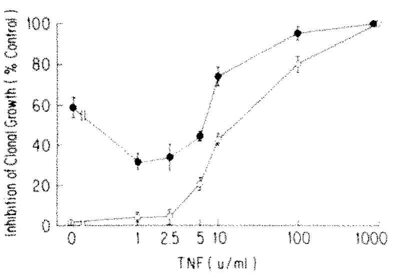

Fig 3. Dose-response curve of the combined effect of TNFα and RA expressed as an inhibition of clonal growth of the myeloblastic leukemic cell line KG-1. Cells were incubated with either TNFα alone (○) or with TNFα and RA 10^{-8} mol/L (●). Data of three independent experiments are expressed as described in the legend of Fig 1A. Control plates containing CSF alone had a mean of 92 to 126 colonies per 3 × 10^3 KG-1 cells.

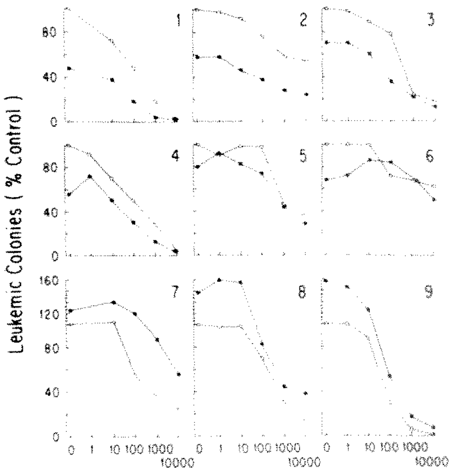

Fig 4. Effect of TNFα and RA on the clonal growth of myeloid cells from nine leukemic patients. The low density mononuclear cells were cultured in the presence of 1.5% Mo-conditioned medium as a source of CSF. TNFα was added either alone (○) or at different concentrations in combination with RA 10^{-7} mol/L (●) to the culture plates. Patients were diagnosed as follows: no. 2, 4, and 5 as CML; no. 3, 7, and 8 as CML in myeloid blast crisis; no. 1 as AML (FAB M2) in relapse; no. 9 as therapy-related AML, no. 6 as CMML. Control dishes containing CSF alone had the following mean numbers of colonies per 2 × 10^5 plated cells: no. 1, 891; no. 2, 46; no. 3, 180; no. 4, 39; no. 5, 469; no. 6, 23; no. 7, 158; no. 8, 206; no. 9, 81.

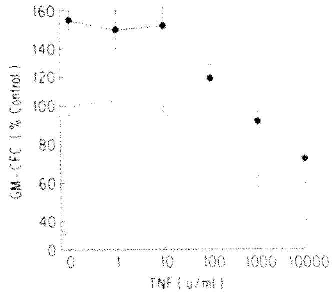

Fig 5. Effect of TNFα and RA on the clonal growth of normal human GM-CFC. Cells were incubated with different concentrations of TNFα without (○) or with RA 10^{-7} mol/L (●) and 1.5% Mo-conditioned medium as a source of CSF. Results are expressed as a mean percentage (±SD) GM-CFC compared with control plates containing CSF alone (no TNFα or RA). Note that RA plus TNFα curve shows the number of GM-CFC in plates containing RA and 0 to 10,000 U/mL TNFα. Control plates having CSF alone had a mean of 167 to 206 colonies per 2 × 10^5 plated bone marrow cells. Each experiment was performed with three different donors in triplicate plates per point.

nearly the same as control plates (mean 92% ± 6% [±SD] of control).

DISCUSSION

Our study shows that TNFα and RA interact in a complex manner with leukemic cell lines, leukemic cells from patients and normal human bone marrow cells. With HL-60 cells, TNFα ($\geq$2.5 U/mL) and RA mainly synergistically inhibited clonal growth (Fig 1A). However, evidence for an antagonism was seen at lower concentrations of TNFα ($\leq$1 U/mL) and RA (10^{-9} mol/L). At these concentration, RA seemed to reduce the expected inhibition of TNFα on clonal growth inhibition of HL-60 cells. The more immature myeloblastic KG-1 cells were less sensitive than HL-60 cells to the inhibitory action of TNFα alone, and only higher concentrations of TNFα ($\geq$10 U/mL) showed a synergistic interaction with RA (Fig 3). At <10 U/mL TNFα, antagonism was seen. A phase I trial with TNFα in cancer patients showed that after intravenous injection of 100 μg/mol/L^2, peak serum levels of 8,000 pg/mL (~300 U/mL) were reached with acceptable toxicity.[30] Our study with HL-60 and KG-1 cells shows that TNFα had synergistic interactions with RA at concentrations of both TNFα and RA that can be achieved in vivo.[30,31]

Recent studies demonstrated that TNFα induced HL-60 cells to differentiate towards monocytes/macrophages.[6,7,10-12] The RA in vitro induced HL-60 and leukemic cells from some patients to differentiate towards granulocytes.[15,16] The ability of the combination of TNFα and RA to enhance differentiation of HL-60 cells (Fig 1B) suggests that part of the synergism between TNFα and RA to inhibit clonal growth might be explained by their combined ability to increase the number of HL-60 cells that differentiate into mature nondividing cells. A previous study by us showed that TNFα preferentially inhibited growth of colonies containing granulocytes rather than macrophages or macrophage/granulocyte colonies.[6] This suggested that the combination of TNFα and RA may have selectively enhanced differentiation along the granulocytic pathway. However, analyses by morphology, cytochemistry and antigen expression showed that the combined treatment with TNFα and RA resulted in differentiation of HL-60 cells along both the macrophage and granulocyte pathway (Table 1), confirming previously reported data.[32]

The TNFα probably mediates its action through binding to specific receptors present on the surface of the cell.[5] Normal and leukemic hematopoietic cells express receptors for TNFα.[7,25,33] The RA (10^{-9} – 10^{-7} mol/L) moderately increased the number of TNFα receptors expressed on HL-60 cells (1.3- to 1.7-fold) as compared with wild-type HL-60 cells, but did not alter the binding affinity of the TNF receptors for their ligand in these cells (Fig 2, Table 2). Studies recently showed that γ-interferon (γ-IFN) increased the number of TNFα receptors on several human carcinoma cell lines, on murine L929 fibroblasts, and on the myeloid U937 cells.[24,33-35] Similar to the combination of TNFα and RA, TNFα plus γ-IFN had an increased cytotoxic/cytostatic effect as compared with each agent alone on human carcinoma cell lines, myeloid leukemia cell lines and leukemic cells from patients,[6,7,11,33,36,37] suggesting that increased cellular expression of the TNFα receptor may explain the enhanced responses. However, recent studies by us as well as others found no direct correlation between the number of receptors expressed on cells of various myeloid lines and their sensitivity to the growth inhibitory effects of TNFα.[7,25,33] Furthermore, TNFα was effective in clonal growth inhibition at concentration well below the saturation of its receptor (<10% occupancy).[7,25,33] Together, these results suggest that expression of increased numbers of TNFα receptors induced by RA is unlikely to account for the greater sensitivity of HL-60 cells and that mechanisms distal to TNFα binding to its receptor might be responsible for the enhanced cytotoxic/cytostatic effect of TNFα in combination with RA.

Various types of responses were observed on clonal growth inhibition of leukemic cells from patients. Both RA and TNFα alone inhibited clonal growth of leukemic cells of the majority of the patients, in agreement with previous studies.[6,13,14] However, RA alone also stimulated clonal growth of three of nine leukemic patients (no. 7 through 9). Two of the patients had CML in myeloid blast crisis (no. 7 and 8), and one had therapy-related AML (no. 9). Similarly, previous studies reported stimulation of leukemic clonal growth in selected patients.[38,39] The combination of TNFα plus RA showed a greater clonal growth inhibition compared with TNFα alone in some patients (no. 1 through 4); whereas in other patients (no. 5 through 9), RA either reduced or even reversed the inhibitory effect of TNFα. This suggests that in selected patients, RA may not enhance the inhibitory effect of TNFα, but possibly may protect leukemic growth from the inhibitory action of TNFα.

Our study confirms previous studies that retinoids enhance in vitro clonal growth of normal human myeloid progenitor cells.[14,18] The TNFα alone inhibited clonal growth of normal human GM-CFC only at much higher concentrations (ED$_{50}$ ~10,000 U/mL) as compared with leukemic clonal growth of both HL-60 cells (ED$_{50}$ 3.5 U/mL) and the majority of clonogenic leukemic cells harvested from patients (Fig 5). The combination of TNFα and RA had less growth inhibitory effect than TNFα alone on normal GM-CFC. This suggests that RA may have a protective effect on the TNFα-mediated inhibition of normal GM-CFC, and theoretically may allow higher concentrations of TNFα to be given without suppressing normal hematopoiesis.

Although RA inhibited in vitro clonal growth of myeloid leukemic cells of the majority of patients, in vivo RA usually had a therapeutic effect limited to patients with acute promyelocytic leukemia[40-43] and selected patients with the myelodysplastic syndrome.[31,44-47] Phase I and II trials with TNFα for AML are ongoing. In vivo animal studies are warranted to investigate further the complex interaction of TNFα and RA on normal and leukemic hematopoiesis and to help identify patients who may benefit from this combined treatment.

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Evidence That Plasma Lipoproteins Inhibit the Factor VIIa-Tissue Factor Complex by a Different Mechanism Than Extrinsic Pathway Inhibitor

By Shinichi Kondo and Walter Kisiel

Factor VIIa participates in blood clotting by activating factor X and/or factor IX by limited proteolysis. The proteolytic activity of factor VIIa is absolutely dependent on a lipoprotein cofactor designated tissue factor. We have examined the ability of purified preparations of human plasma high density, low density and very low density lipoproteins, as well as apolipoproteins A-I and A-II, to inhibit the factor VIIa-tissue factor mediated activation of either factor X or factor IX before and after treatment of the lipoprotein preparation with polyclonal antibody directed against partially-purified human plasma extrinsic pathway inhibitor (EPI). In the absence of anti-EPI IgG, HDL, LDL, VLDL, and apolipoprotein A-II noncompetitively inhibited factor X activation by factor VIIa-tissue factor with apparent Ki values of 3.39 µmol/L, 124 nmol/L, 33 nmol/L, and 10.5 µmol/L, respectively. Apolipoprotein A-I had no effect on this reaction. The inhibitory activity of HDL, LDL, VLDL, and apolipoprotein A-II in this reaction was unaffected by the presence of high levels of anti-EPI IgG. In the absence of exogenous factor Xa, none of the lipoproteins studied inhibited the activation of factor IX using the tritiated peptide release assay. In the presence of added factor Xa (1 nmol/L), LDL and VLDL, but not HDL and apolipoprotein A-II, inhibited the activation of factor IX by factor VIIa-tissue factor. This inhibition was completely blocked by prior incubation of the lipoprotein with anti-EPI IgG indicating association of EPI with these particles. Taken collectively, our data indicate that HDL, LDL, and VLDL, at or below their plasma concentration, each selectively inhibits the factor VIIa-tissue factor mediated activation of factor X by a mechanism that appears to be distinct from extrinsic pathway inhibitor. These lipoproteins may not only play a role in the regulation of extrinsic blood coagulation, but may also selectively promote the activation of factor IX by factor VIIa-tissue factor in vivo at low tissue factor concentrations.

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FACTOR VII is a trace, vitamin K-dependent glycoprotein that circulates in blood as a zymogen to a serine protease, factor VIIa. Single chain human factor VII is converted to factor VIIa through the cleavage of a single internal peptide bond located at Arg$_{152}$-Ile$_{153}$.[1] The resultant two-chain factor VIIa activates either factor X or factor IX only in the presence of a lipoprotein cofactor designated tissue factor. In contrast to the three other activated vitamin K-dependent coagulation factors (factor IXa, factor Xa, and thrombin), factor VIIa is not effectively inhibited by antithrombin III.[2-5] The physiological mechanism whereby the factor VIIa-tissue factor complex is regulated in hemostasis is poorly understood.

In 1981, Carson[6] reported that plasma high density lipoproteins inhibit the activation of factor X by factor VIIa-tissue factor. More recently, several laboratories[7-12] have identified a novel mechanism for the inhibition of factor VIIa-tissue factor involving the copresence of factor VIIa, tissue factor, factor Xa, and a 35 to 40 kilodaltons (kDa) plasma protein initially isolated from a plasma lipoprotein fraction[8-10] and more recently purified to homogeneity from serum-free conditioned medium from Hep G-2 cells.[11-12] In this mechanism, extrinsic pathway inhibitor (EPI) presumably recognizes a ternary complex of factor VIIa-tissue factor-factor Xa and inhibits further activation of factor X and/or factor IX.[9,12] In addition, the active site of factor Xa appears to be essential for this inhibition to occur.[9,12] The association of EPI with the plasma lipoprotein fraction suggested that Carson's data concerning the inhibition of factor VIIa-tissue factor by HDL may have resulted, in large part, from contamination by EPI.[9] However, a recent report by Carson indicates that the inhibitory activity of HDL resides totally in the apolipoprotein A-II portion of this lipoprotein.[13] In the latter study, apolipoprotein A-II was purified to homogeneity by gel permeation column chromatography in the presence of 3 mol/L guanidine hydrochloride, a procedure that would in all likelihood resolve the apolipoprotein from contaminating EPI. In this report, we show that purified preparations of human plasma HDL, LDL, VLDL, and apolipoprotein A-II inhibits the factor VIIa-tissue factor mediated activation of factor X with Ki values at or below their respective plasma concentrations. None of these lipoprotein preparations demonstrated inhibitory activity against factor VIIa-tissue factor when factor IX was used as the substrate in the absence of added factor Xa. In the presence of factor Xa, however, LDL and VLDL demonstrated inhibitory activity with factor IX as the substrate and this activity was completely abolished following incubation of the lipoprotein with immunoglobulin raised against partially-purified EPI. In contrast, treatment of each lipoprotein (HDL, LDL, and VLDL) with this antibody had no effect on the ability of the lipoprotein to inhibit the activation of factor X by a complex of factor VIIa-tissue factor.

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MATERIAL AND METHODS

Materials. Bz-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) was obtained from Helena Laboratories, Beaumont, TX. Sodium dodecyl sulfate (SDS) was obtained from British Drug House, Poole, England. Bovine serum albumin (fatty acid-free), Tris (Trizma base), potassium bromide, polyoxyethylene sorbitan monolaurate (Tween 20), Triton X-100, and sodium deoxycholate were obtained from Sigma, St Louis. Acrylamide, N, N'-methylene bisacrylamide, N, N, N', N'-tetramethylene diamine, Coomassie Brilliant Blue G-250, Protein Assay Dye Reagent Concentrate, Affi-Gel 10, and Affi-Gel 15 were products of Bio-Rad, Richmond, CA. Sodium Boro [^{3}H] hydride (21.6 C/m mol) was purchased from Amersham Corporation, Arlington Heights, IL. Rabbit anti-human apolipoprotein B antiserum was obtained from Calbiochem-Behring, La Jolla, CA. Sheep anti-human apolipoprotein A-I and sheep anti-human apolipoprotein A-II were purchased from Boehringer Mannheim, Indianapolis. SDS High Mr protein standard kit was a product of Pharmacia, Piscataway, NJ. All other chemicals were the best grade available from commercial sources.

Human brain cephalin (mixed phospholipids) was prepared according to Bell and Alton.[14] Human brain tissue factor apoprotein was prepared essentially according to Broze et al.[15] In this procedure, 25 g of human brain acetone powder were resuspended in 500 mL of 0.05 M Tris-HCl (pH 7.5) containing 0.1 mol/L NaCl, 0.1% NaN$_3$, 5 mmol/L EDTA, and 2 mmol/L DFP and stirred for one hour at room temperature. The mixture was centrifuged at 12,000 g for 1.5 hours at 20°C. The supernatant was discarded and the pellet was resuspended in 500 mL TBS/2% Triton X-100/0.1% NaN$_3$ and stirred at room temperature for one hour. This suspension was centrifuged at 30,000 g at 4°C for 75 minutes. The supernatant was adjusted to 10 mmol/L CaCl$_2$, stirred at room temperature for 30 minutes, and incubated overnight at 4°C. A precipitate that formed during incubation was removed by centrifugation at 40,000 g for one hour at 4°C, and the supernatant subsequently concentrated by ultrafiltration to ~40 mL. The crude tissue factor concentrate was again centrifuged (48,000 g; one hour; 4°C) and the pellet discarded. The supernatant was applied at room temperature to tandem columns of glycine-ethyl ester-Affi-Gel (2.6 × 5 cm) and human factor VII-Affi-Gel (1.6 × 2 cm) at a flow rate of 10 mL/h. Following a wash of both columns with 50 mL of TBS/10 mmol/L CaCl$_2$/2% Triton X-100, the glycine ethyl ester-Affi-Gel column was removed and the factor VII-Affi-Gel column was eluted with TBS/20 mmol/L EDTA/2% Triton X-100. Fractions containing tissue factor activity were pooled and stored at 4°C. Using this procedure, ~80% of the tissue factor activity eluted in the unadsorbed fraction of factor VII-Affi-Gel column, while the remaining 20% was recovered in the EDTA-eluant. The low yield of purified TF apoprotein was consistently observed whether factor VII (15 mg) was coupled to 4 mL of Affi-Gel 15 or 4 mL activated CH-Sepharose (Pharmacia). Examination of affinity-purified human tissue factor apoprotein by SDS-PAGE revealed two major bands with mol wt values of 61,000 and 47,000 in the presence or absence of reducing agents. As revealed by gel slicing experiments, tissue factor coagulant activity was confined exclusively to the 47,000 Mr band. The affinity-purified tissue factor apoprotein was relipidated as described[15] with the exception that human brain mixed phospholipids were used instead of rabbit brain phospholipids.

A DEAE Affi-Gel Blue purified rabbit immunoglobulin preparation that neutralized extrinsic pathway inhibitor activity was generously provided by Drs Warn-Cramer and Rapaport, University of California, San Diego. The EPI antigen used for immunization was purified ~1000-fold from the total lipoprotein fraction of human plasma by a combination of gel filtration, ion exchange chromatography and preparative SDS-PAGE, and contained both Mr variants of EPI, significant amounts of apolipoprotein E and minor amounts of apolipoprotein A-II and apolipoprotein A-IV (S. Rapaport, personal communication, December, 1986). Anti-EPI IgG formed a single, faint precipitin band in Ouchterlony double diffusion experiments when allowed to react with either a purified preparation of HDL or apolipoprotein A-II, but failed to form any detectable precipitin band when allowed to react with various concentrations of either LDL, VLDL, or apolipoprotein A-I.

Human factor X, factor VII, and factor IX were partially purified from therapeutic plasmapheresis plasma by a combination of barium citrate adsorption and elution and DEAE-Sepharose CL-6B chromatography essentially as described.[16] Factor VII obtained from DEAE-Sepharose chromatography was purified to homogeneity by immunoaffinity chromatography as described.[1] Single-chain factor VII was activated to two-chain factor VIIa by factor Xa as described.[1] The factor X pool obtained from DEAE-Sepharose chromatography was further purified on a column of dextran sulfate agarose (5.0 × 45 cm) equilibrated at 4°C with 0.025 mol/L sodium citrate (pH 7.4)/1 mmol/L benzamidine. Proteins were eluted in a linear NaCl gradient composed of 2 L of equilibrating buffer and 2 L 0.025 mol/L sodium citrate (pH 7.4)/1M NaCl/1 mmol/L benzamidine. This DSA column effectively resolved factor X from protein S, prothrombin, protein C, and factor IX. The factor X obtained from DSA chromatography was homogeneous as judged by SDS-PAGE. However, this preparation was found to be contaminated with low density lipoproteins and high density lipoproteins as evidenced by positive immunoprecipitates with monospecific antisera against apolipoprotein B and apolipoprotein A-I/A-II. Consequently, the DSA-factor X pool was purified to homogeneity by preparative polyacrylamide gel electrophoresis as described elsewhere.[17] Alternatively, lipoproteins were effectively separated from DSA factor X by gel filtration chromatography in Sephadex G-150 equilibrated with TBS/1 mmol/L Benzamidine. Both preparations of purified factor X appeared as single bands on 10% SDS-gels, and were negative for immunoreactive apolipoprotein B and apolipoprotein A-I/A-II. Factor X was activated by incubation with an insolubilized preparation of RVV-X as described.[18] The factor IX obtained from DSA chromatography was separated from contaminating lipoproteins and purified to homogeneity by immunoaffinity chromatography using a column containing murine monoclonal antibody to human factor IX (A-7) coupled to Affi-Gel 10.[19] The affinity column was equilibrated with 0.02 mol/L Tris-HCl (pH 7.2)/0.15 mol/L NaCl/20 mm MgCl$_2$/1 mmol/L benzamidine and the adsorbed factor IX was eluted from the column with 0.02 mol/L Tris-HCl (pH 7.2)/0.15 mol/L NaCl/1 mmol/L benzamidine/20 mmol/L EDTA. Human factor VII (14.6 mg) was coupled to 4 mL packed volume of Affi-Gel 15 at RT for two hours. The coupling reaction was performed in 0.1 mol/L Hepes (pH 7.5), and residual, unreacted sites on the Affi-Gel 15 were subsequently blocked with glycine ethyl ester (0.1 mol/L final concentration).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS-PAGE was performed according to Laemmli[20] using a 2.5% concentrating gel and a 3% to 15% linear gradient polyacrylamide resolving gel. Gels were stained for one hour in 0.1% Coomassie Blue G-250/50% methanol/10% acetic acid and diffusion destained in 10% methanol/10% acetic acid.

Various assays. Human factor VII, factor X, and factor IX were assayed by standard one-stage assays using hereditary deficient plasmas as substrates.[3,21,22] The coagulant activity of relipidated tissue factor apoprotein was measured by incubating 100 μL of diluted tissue factor with 100 μL of normal pooled human plasma for 30 seconds, followed by the addition of 100 μL of 25 mmol/L calcium chloride. In this system, 2 ng of relipidated tissue factor apoprotein produced a clot in ~50 seconds. The protein concentrations of factor X, factor VII, factor VIIa, tissue factor apoprotein, and purified plasma lipoproteins were determined by the dye binding assay as described by Bradford[23] using bovine serum albumin as the reference protein.

The inhibition of the factor VIIa-tissue factor mediated activation of factor X by plasma lipoproteins was determined in a modified two-stage amidolytic assay originally described by Seligsohn et al.[24] In this assay, 50 μL of tissue factor (0.34 nmol/L in apoprotein) was incubated for 15 minutes in a polystyrene tube at 37°C with 50 μL of either buffer control (TBS/BSA) or various concentrations of lipoproteins in TBS/BSA. To this system was added 50 μL of factor VIIa (0.16 nmol/L), 50 μL of human factor X (27 to 228 nmol/L) and 25 μL of calcium chloride (50 mmol/L). Incubation at 37°C was continued for exactly one minute after the addition of $CaCl_2$, and at this time 25 μL of EDTA (300 mmol/L) was added to stop the activation reaction. One hundred microliters of the incubation mixture was removed and added to 700 μL of S-2222 (192 μmol/L) in 0.05 mol/L Tris-HCl/0.15 mol/L NaCl (pH 8.3) in a cuvette. The absorbance at 405 nm was continuously recorded using a Beckman Model 25 spectrophotometer equipped with a strip chart recorder. Factor Xa concentrations were interpolated from a standard curve of ΔA_{405}/min *v* factor Xa concentration (Fig 1).

The inhibition of the factor VIIa-tissue factor mediated activation of factor IX by plasma lipoprotein was determined in a modified tritiated activation peptide release assay originally described by Zur and Nemerson.[25] This assay was constructed the same as the chromogenic assay except the tritiated factor IX (21 to 803 nmol/L) was substituted for factor X and the final incubation volume was increased to 450 μL. At appropriate intervals, 100 μL subsamples of the incubation mixture were transferred to polypropylene tubes containing 200 μL of 50 mmol/L EDTA/50 mmol/L benzamidine/TBS and kept on ice. At the end of the incubation period, 150 μL of ice-cold 15% trichloracetic acid (TCA) was added to each subsample and this mixture centrifuged five minutes in a Beckman microfuge B. Duplicate samples of each supernatant were counted in a Packard Tri-Carb scintillation counter. In our system, the factor IX activation progress curve plateaued when 31% of the total counts were released as TCA soluble material. We assume that this level of TCA soluble counts represents complete activation of [^3H]-factor IX, and used this value to calculate the moles of factor IX temporally activated.

Tritium labeled factor IX was prepared by the modification of Van Lenten and Ashwell.[26] In our hands, the specific radioactivity of tritiated factor IX was 0.9 × 10^5 cpm/μg, and the biological activity of labeled factor IX was 67% of nonlabeled preparations. Kinetic parameters were determined from three individual experiments using the nonlinear, nonweighted, least square fitting to a hyperbola.[27]

The molar concentrations of plasma lipoproteins used in this assay were calculated from the observed protein mass concentration by the dye-binding assay,[13] and from published values of the weight percentage of apolipoprotein in each lipoprotein[28] as well as the mol wt of the intact lipoprotein.[29] In the case of VLDL, where ranges of mol wt are reported (10 to 80 × 10^6), a mean value was assumed. Specific mol wt values used in this study for VLDL, LDL, and HDL were 50 × 10^6, 2.3 × 10^6, and 1.75 × 10^5 Da, respectively.

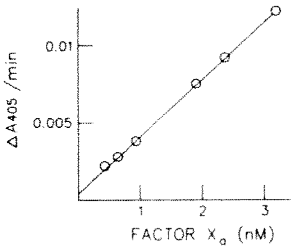

Fig 1. **Human factor Xa amidolytic assay standard curve.**

Isolation of plasma lipoproteins and apolipoproteins A-I and A-II. HDL, LDL, and VLDL were isolated from plasma obtained from a fasted normolipemic donor by sequential preparative ultracentrifugation essentially as described by Havel et al.[30] Ultracentrifugation was carried out in a Beckman model L5-75 preparative ultracentrifuge at 15°C. Briefly, nine parts of blood were mixed with one part of an anticoagulant cocktail containing 0.13 mol/L sodium citrate, 0.02% sodium azide, and 50 mmol/L EDTA. Following centrifugation to remove blood cells, the plasma was adjusted to 1 mmol/L in PMSF and 10 mmol/L benzamidine. Chylomicrons were removed from plasma by centrifugation at 78,000 g for 150 minutes in an SW-40 rotor. Subsequently, the VLDL was floated following centrifugation of the infranate at 105,000 g for 22 hours in a 50 Ti fixed angle rotor. LDL and HDL were floated sequentially after density adjustment of the infranates to 1.063 and 1.21, respectively, by the addition of either stock salt solution (NaCl/KBr; density = 1.346) or solid KBr.[30] LDL was floated at 105,000 g for 22 hours, while HDL was floated at 105,000 g for 48 hours. VLDL was refloated at least one time at d = 1.0063 g/mL by centrifugation for 22 hours at 105,000 g. LDL was refloated sequentially at d = 1.063 g/mL and d = 1.050 g/mL to remove contaminating HDL. Despite recentrifugation at d = 1.21 g/mL, the HDL preparation revealed the presence of contaminating apolipoprotein B by immunoassay that could not be removed by readjustment of the HDL solution to d = 1.063 (by dialysis) and subsequent refloatation. Accordingly, low levels of immunoreactive LDL were quantitatively removed from the HDL preparation by passage of the HDL through a column of rabbit anti-Apo B-IgG-protein A-Sepharose. Each purified lipoprotein preparation was dialyzed extensively against TBS/0.02%

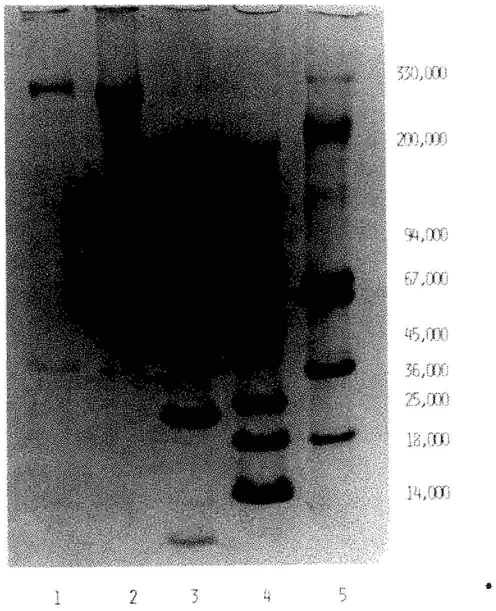

Fig 2. **SDS-PAGE of human plasma lipoproteins on a 3% to 15% polyacrylamide gradient gel. Lanes: 1, VLDL, 6 μg; 2, LDL, 8 μg; 3, HDL, 15 μg; 4, mol wt standards (Myosin, 200,000; phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300); 5, mol wt standards (thyroglobulin, 330,000; ferritin, 220,000; albumin, 67,000; catalase, 60,000; lactic dehydrogenase, 36,000; apoferritin, 18,500). All samples were electrophorized after reduction with 10% 2-mercaptoethanol.**

NaN_3 and stored at 4°C. When examined by SDS-PAGE,[20] unreduced preparations of VLDL exhibited a major band at 300 kd, LDL a major band at 280 kd, and HDL a major band at 24 kd (Apo A-I) and a minor band at 15 kd (Apo A-II). Reduction with 5% 2-mercaptoethanol had no effect on the Mr of VLDL or LDL (Apo B), whereas the 15 kd band in HDL decreased to ~7 kd. The 24 kd (Apo A-I) band was also unaffected by reduction (Fig 2). By Ouchterlony double diffusion analysis, the VLDL and LDL preparations appeared immunologically pure and showed no precipitation band against rabbit anti-apolipoprotein A-I/A-II. Similarly, the HDL preparations were free of immunoreactive apolipoprotein B, and formed a single precipitin arc against rabbit anti-apolipoprotein A-I/A-II.

A mixture of apolipoproteins A-I/A-II was isolated from HDL by the following procedure. Two milliliters of HDL (1.22 mg/mL) were dialyzed exhaustively against 50 mmol/L Tris-HCl (pH 7.5) and subsequently lyophilized. The lyophilized lipoprotein (plus Tris) was extracted four times with 15 mL of chloroform: methanol (2:1) at 0°C (ice-water bath). The final pellet was dried under N_2 and solubilized in 1.5 mL TBS/40 mmol/L sodium deoxycholate (DOC). This solution was diluted with 50 mL TBS in order to reduce the DOC concentration to below its critical micelle concentration (0.9 mmol/L)[31] and the resulting solution dialyzed exhaustively against TBS at 4°C. The retentate was subsequently concentrated to 2 mL by ultrafiltration (YM-10 membrane). Apolipoproteins A-I and A-II were isolated as described.[32] Shortly after delipidation by chloroform:methanol (2:1), the HDL was dissolved in 3 mol/L guanidine hydrochloride/TBS and applied to Sephacryl S200 column chromatography (1.5 × 170 cm) equilibrated with the same buffer. Column chromatography was repeated once to achieve homogeneity (Fig 3). Purified apolipoproteins were dialyzed against TBS/0.02% NaN_3 and concentrated by ultrafiltration (YM10; Amicon, MA).

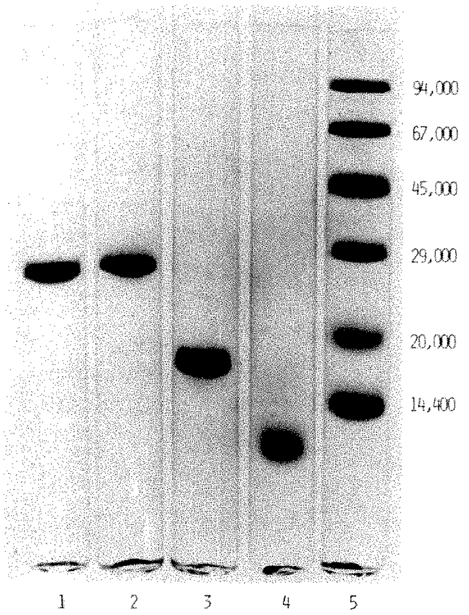

Fig 3. SDS-PAGE of human apolipoprotein A-I and A-II. Lanes: 1, nonreduced Apo A-I, 12 μg; 2, reduced Apo A-I, 12 μg; 3, nonreduced Apo A-II, 14 μg; 4, reduced Apo A-II, 14 μg; 5, mol wt standards (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; soybean trypsin inhibitor, 20,000; α-lactalbumin, 14,400).

RESULTS

Validity of the coupled amidolytic assay. The two stage coupled amidolytic assay was used to determine the inhibitory effect of purified plasma lipoproteins on factor Xa generation by a complex of factor VIIa and tissue factor. The assay was standardized with respect to tissue factor concentration, factor X concentration, factor VIIa concentration and incubation time such that the rate of factor Xa generation was first-order and linear. In our system, factor Xa production was linear between factor VIIa concentrations of 2.5 and 35 pmol/L and tissue factor concentrations between 6.8 and 78 pmol/L during a one-minute incubation period. At these reactant concentrations, linear production of factor Xa was consistently preceded by a lag period of ~30 seconds similar to that reported by Silverberg et al[33] using the radiolabeled activation peptide release assay. Tissue factor was routinely incubated with either lipoproteins or apolipoproteins for 15 minutes at 37°C before the addition of factor VIIa, factor X, and $CaCl_2$; however, incubation times of two to 15 minutes produced the same effect. The tissue factor and factor VIIa final concentrations selected for these experiments were 75 pmol/L and 35 pmol/L, respectively. The tissue factor concentration actually represents the molar concentration of tissue factor apoprotein assuming a mol wt of 47,000 Da. As mentioned earlier, our tissue factor apoprotein preparation revealed two bands by SDS-PAGE with mol wt values of 61,000 and 47,000 in roughly a 40:60 ratio (61,000:47,000) as judged from densitometry scans of the gels. To simplify our calculations, we are assuming that the 61,000 Mr species represents a contaminant and the concentration of tissue factor apoprotein has been expressed as the amount of the 47,000 Mr species by multiplying the total protein concentration by a factor of 0.6. The factor X preparation was homogeneous by SDS-PAGE and was free of lipoproteins, factor IX, factor Xa, and factor VII. No amidolytic activity could be detected when factor X was incubated with calcium in the presence or absence of tissue factor. In preliminary experiments designed to compare the activation of gel-filtered factor X *v* factor X purified by preparative electrophoresis, a significantly higher (twofold) Vmax was obtained using gel-filtered factor X. In addition, the specific coagulant activity of gel-filtered factor X was threefold higher than electrophoresis factor X (106 U/mg *v*

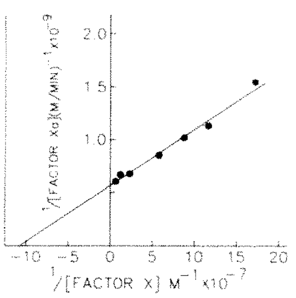

Fig 4. Lineweaver-Burk plot of the activation of factor X by factor VIIa-tissue factor. The range of factor X final concentrations used was 6 to 200 nmol/L.

30 U/mg) in an assay using RVV-X to activate the factor X. Accordingly, gel-filtered factor X was used for the inhibition studies using the amidolytic assay.

Using the above conditions, the kinetic parameters of the activation of human factor X by a complex of human factor VIIa-tissue factor were determined (Fig 4). The Km is 10.49 ± 4.88 nmol/L factor X. The Vmax is 1.67 ± 0.19 nmol l^{-1} min^{-1}, which yields a turnover number (Kp) of 50 $minutes^{-1}$. These kinetic parameters are in reasonably good agreement with those recently published for the activation of bovine factor X by purified preparations of bovine factor VIIa and bovine brain tissue factors.[34]

Construction of the peptide release assay. The peptide release assay for factor IX activation was constructed similar to the chromogenic assay for factor X activation. Tritium labeled factor IX activation peptide production was linear between factor VIIa concentrations of 4 to 27 pmol/L and tissue factor concentrations between 20 and 112 pmol/L up to 15 minutes incubation time.

The tissue factor and factor VIIa concentration selected for the inhibition experiments was 112 pmol/L and 27 pmol/L, respectively. The kinetic parameters for the activation of human factor IX by a complex of human factor VIIa-tissue factor are as follows: Km, 47.44 ± 24.76 nmol/L factor IX; Vmax, 0.439 ± 0.055 nmol l^{-1} min^{-1}, yielding a turnover number (Kp) of 16 min^{-1} (Fig 5).

Inhibition of factor X activation by purified plasma lipoproteins and apolipoproteins. Factor X activation was measured over the concentration range of 6 to 50 nmol/L factor X in the presence of varying levels of purified HDL, LDL, and VLDL. In control experiments where tissue factor was replaced with buffer, no activation of factor X was observed by factor VIIa in the presence of various concentrations of each lipoprotein. Each lipoprotein had no effect on factor Xa amidolytic activity in the range used to inhibit the factor VIIa-tissue factor catalyzed activation of factor X. In addition, each lipoprotein had no effect on the duration of the lag period preceeding the linear production of factor Xa in each system.

The results for the inhibition of factor X activation by HDL are shown in Fig 6 as a Dixon plot. The type of inhibition observed was distinctly noncompetitive, and the apparent inhibitor constant (Ki) for HDL was 3.39 μmol/L.

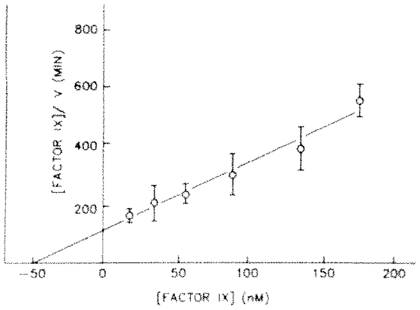

Fig 5. Hanes-Woolf plot of the activation of factor IX by factor VIIa-tissue factor. The range of factor IX final concentrations used was 14 to 179 nmol/L.

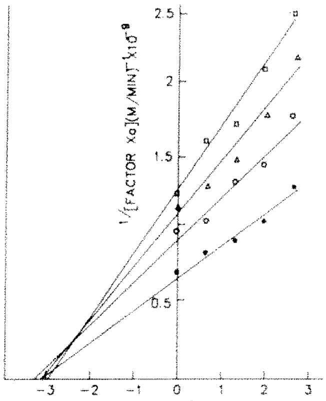

Fig 6. Dixon plot for the inhibition of factor X activation by HDL. The rate of factor X activation was determined in the coupled amidolytic assay as described in Materials and Methods. The factor X final concentrations used were: (●), 47.9 nmol/L; (○), 19.2 nmol/L; (△), 12.8 nmol/L; (□), 9.6 nmol/L.

The inhibition of factor X activation by LDL and VLDL also exhibited a noncompetitive type of inhibition. Ki values of 124 nmol/L and 33 nmol/L were obtained from Dixon plots (Figs 7 and 8) for LDL and VLDL, respectively. Delipidated HDL, containing a mixture of apolipoproteins A-I and A-II, exhibited the same type of inhibition (Ki = 5.5 μmol/L) and was essentially as effective as intact HDL in the inhibition of factor X activation (Table 1). Comparable experiments to assess the inhibitory potential of apolipoprotein B failed due to the insolubility of apolipoprotein B following chloroform:methanol extraction of LDL. The inhibitory activity, observed in a mixture of apolipoproteins A-I/A-II was subsequently shown to be due entirely to apolipoprotein A-II. Each apolipoprotein was purified to homogeneity by repetitive gel-filtration column chromatography in the presence of 3 mol/L guanidine hydrochloride. Purified apolipoprotein A-I failed to show any factor X activation inhibitory activity in our system at concentrations as high as 100 μmol/L (Table 1) whereas the apolipoprotein A-II noncompetitively

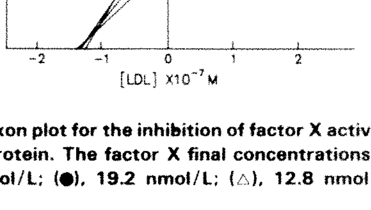

Fig 7. Dixon plot for the inhibition of factor X activation by low density lipoprotein. The factor X final concentrations used were: (○), 47.9 nmol/L; (●), 19.2 nmol/L; (△), 12.8 nmol/L; (▲), 9.6 nmol/L.

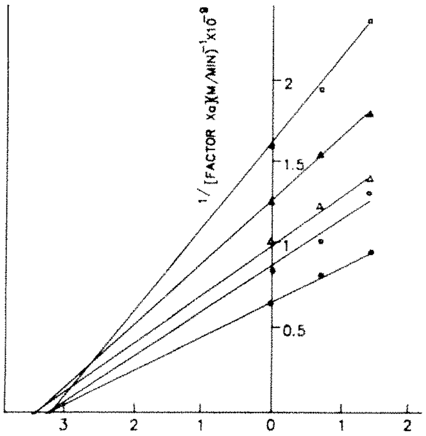

Fig 8. Dixon plot for the inhibition of factor X activation by very low density lipoprotein. The factor X final concentrations used were: (●), 47.9 nmol/L; (○), 19.2 nmol/L; (△), 12.8 nmol/L; (▲), 9.6 nmol/L; (□), 6.4 nmol/L.

inhibited the factor X activation reaction with an apparent Ki value of 10.5 μmol/L (Fig 9). The results of the inhibition of factor X activation by each lipoprotein and soluble apolipoprotein are shown in Table 1. The Ki values for each lipoprotein appear to be at or below their plasma concentration, assuming plasma concentrations of 13 μmol/L, 1.5 μmol/L, and 28 nmol/L for HDL, LDL, and VLDL, respectively.[29]

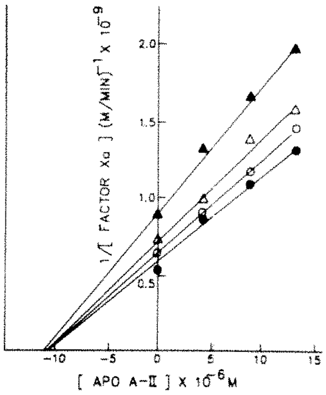

Fig 9. Dixon plot for the inhibition of factor X activation by apolipoprotein A-II. The rate of factor X activation was determined in the coupled amidolytic assay as described in Methods. The factor X final concentrations used were: (●), 68 nmol/L; (○), 45 nmol/L; (△), 34 nmol/L; (▲), 23 nmol/L.

Inhibition of factor IX activation by purified plasma lipoproteins and apolipoproteins. The activation of [^{3}H]-factor IX by a complex of factor VIIa-tissue factor was measured over the concentration range of 14 to 179 nmol/L factor IX in the presence of varying levels of two different preparations of lipoproteins and apolipoproteins. The results of quadruplicate measurements at a factor IX concentration of 179 nmol/L are shown in Table 2 for one preparation of each lipoprotein and apolipoprotein. In comparison with a control mixture, none of the lipoproteins or apolipoproteins tested inhibited this reaction in the absence of added factor Xa. One preparation of apolipoprotein A-II, however, produced a slight enhancement (twofold) of the rate of factor IX activation that appeared to be statistically significant (data not shown). This effect was not observed when factor X was the substrate using this particular apolipoprotein A-II preparation. Furthermore, the reason for the stimulating effect on the factor IX activation by this particular apolipoprotein A-II preparation is not known and additional studies are required to determine the reproducibility of this effect with several different preparations of apolipoprotein A-II. When the activation of factor IX by factor VIIa-tissue factor was conducted in the presence of added factor Xa (1 nmol/L final concentration), both VLDL and LDL inhibited this reaction suggesting the presence of EPI associated with these particles. Pretreatment of VLDL and LDL preparations with increasing concentrations of anti-EPI–IgG completely abolished the ability of VLDL and LDL to inhibit the activation of factor IX (Fig 10). Interestingly, these same incubation mixtures of VLDL–anti-EPI and LDL–anti-EPI inhibited the factor VIIa-tissue factor mediated activation of factor X using final concentrations of factor VIIa and tissue factor essentially identical to that used for the activation of factor IX (Fig 10). The slight recovery of inhibitory activity observed for LDL and VLDL in the factor IX activation system (Fig 10) probably reflects a condition of antibody excess relative to EPI concentration and the consequent solubilization of the antigen-antibody complex. Thus, these data strongly suggest that the apparent inhibition of factor IX activation by purified preparations of LDL and VLDL result from contaminating EPI in these lipoprotein preparations. In both cases, the expression of this inhibitory activity was dependent on the presence of exogenous factor Xa. In contrast, purified preparations of HDL and apolipoprotein

Table 1. Inhibition Constants of Plasma Lipoproteins and Apolipoproteins for the Activation of Factor X by Factor VIIa-Tissue Factor

Lipoprotein/Apolipoprotein	Ki
VLDL	33 nmol/L
LDL	124 nmol/L
HDL	3.4 μmol/L
Delipidated HDL	5.5 μmol/L
Apo A-I	>100 μmol/L
Apo A-II	10.5 μmol/L

Table 2. Effect of Purified Lipoproteins and Apolipoproteins on Factor IX Activation by Factor VIIa-Tissue Factor in the Presence and Absence of Added Factor Xa

Lipoprotein/ Apolipoprotein	Concentration (μmol/L)	Rate (nmol l^{-1} min^{-1}) (−Xa)	Rate (nmol l^{-1} min^{-1}) (+Xa)
Control	—	0.321 ± 0.046	0.292 ± 0.038
VLDL	0.024	0.298 ± 0.030	0.156 ± 0.039
LDL	0.185	0.329 ± 0.032	0.057 ± 0.015
HDL	2.7	0.294 ± 0.033	0.280 ± 0.068
Apo A-I	6.7	0.282 ± 0.075	0.294 ± 0.038
Apo A-II	24.8	0.297 ± 0.029	0.281 ± 0.040

Factor IX concentration, 179 nmol/L; factor Xa concentration, 1 nmol/L.

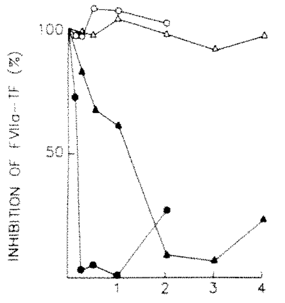

Fig 10. The effect of anti-EPI–IgG on the ability of LDL and VLDL to inhibit factor VIIa-tissue factor. LDL (△,▲) and VLDL (○,●) at a fixed concentration was incubated with increasing concentrations of anti-EPI–IgG for two hours at 37°C. An aliquot of this incubation mixture was tested for its ability to inhibit the activation of either factor X (open symbols) or factor IX (closed symbols) as described in Materials and Methods. Factor IX activation incubation mixture also contained 1 nmol/L factor Xa. The inhibition observed by each lipoprotein in the absence of anti-EPI–IgG (buffer control) was designated as the 100% value. LDL final concentration in each activation reaction was 150 nmol/L; VLDL concentration in each activation reaction was 40 nmol/L.

A-II, as well as LDL and VLDL preparations previously treated with immunoglobulin that neutralized EPI activity, markedly and specifically inhibited the activation of factor X by a complex of factor VIIa-tissue factor without demonstrating any apparent effect on the ability of factor VIIa-tissue factor to activate factor IX.

DISCUSSION

We have carried out experiments to assess the inhibitory properties of purified plasma lipoproteins on the activation reactions of factor X and factor IX by a complex of purified factor VIIa and human brain tissue factor. Our data clearly indicate that HDL, LDL, and VLDL effectively inhibit the activation of factor X by factor VIIa-tissue factor at or below their respective plasma concentrations (Table 1). Of these three lipoproteins, given their plasma concentrations and Ki values, the most effective inhibitor of this reaction would appear to be LDL.

With regard to HDL, our data confirm earlier reports by Carson that described inhibition of the bovine factor VIIa-tissue factor catalyzed activation of bovine factor X by purified human HDL and apolipoprotein A-II.[6,13] Both our data, and that of Carson,[13] suggest that a specific interaction between the Apo A-II and tissue factor occurs resulting in allosteric modifications of the tissue factor molecule.

Inasmuch as factor VIIa-tissue factor also activates factor IX in the test tube,[35] we examined whether or not the above lipoprotein preparations could inhibit the activation of factor IX at approximately the same enzyme-cofactor–substrate concentrations. Under these conditions, none of the lipoprotein preparations inhibited the activation of [^{3}H]-factor IX when present at a concentration that inhibited the factor X activation by 50%. When these incubation mixtures were supplemented with purified factor Xa (1 nmol/L), appreciable inhibition of factor IX activation was observed for the LDL and VLDL preparations. This factor Xa-dependent inhibition was, however, abolished on preincubation of LDL and VLDL with antibody raised against partially purified extrinsic pathway inhibitor. To our complete surprise, preincubation of LDL and VLDL with anti-EPI had no detectable effect on the ability of these lipoproteins to inhibit the activation of factor X by factor VIIa-tissue factor. Thus, these data provide suggestive evidence that LDL and VLDL inhibit the activation of factor IX by factor VIIa-tissue factor as a result of EPI associating and copurifying with these lipoprotein particles. On the other hand, HDL, LDL, and VLDL preparations appear to inhibit the activation of factor X by factor VIIa-tissue factor by a mechanism distinct from extrinsic pathway inhibitor. The precise mechanism whereby these lipoproteins selectively affect factor X activation without affecting factor IX activation is completely unknown. Presumably any proposed mechanism that involves either apolipoprotein exchange between lipoprotein and tissue factor, or the formation of a nonproductive complex between factor VIIa and lipoprotein would affect both reactions to the same degree. Furthermore, assuming our LDL and VLDL preparations were contaminated by EPI, we have no explanation as to why the inhibition of factor X activation by these lipoproteins was not diminished to any significant extent by prior incubation with anti-EPI–IgG. Presumably, sufficient nascent factor Xa was produced in our factor X activation system in a one-minute incubation period to form the factor VIIa-tissue factor-factor Xa ternary complex recognized by EPI present in the LDL and VLDL preparations prior to antibody treatment. Alternatively, in the event that the formation of this ternary complex was not instantaneous, the expression of EPI inhibitory activity may not have occurred under our experimental conditions. The possibility of a negative feedback reaction involving proteolysis of factor VIIa by newly formed factor Xa may play some role in the apparent inhibition of this reaction and cannot be excluded. However, in our experience, little, if any, proteolytic degradation of human factor VIIa by factor Xa was observed at a 1:100 factor Xa:factor VII ratio for one hour in the presence of mixed phospholipids and calcium (5 mmol/L). Whether or not degradation of factor VIIa by factor Xa is enhanced in the presence of tissue factor and/or lipoproteins remains to be determined.

Our findings, in addition to earlier reports[6,13] clearly underline the effectiveness of plasma lipoproteins to inhibit the activation of factor X in the test tube. Whether or not these reactions occur to any significant extent in vivo is unknown. Given the fact that the extrinsic pathway does not appear to be operative to any significant extent in hemophilic patients, the activation of factor IX by factor VIIa-tissue factor may be a very significant reaction in hemostasis. Indeed, experimental evidence indicates that the activation of factor X and factor IX by factor VIIa-tissue factor in human plasma proceeds at approximately the same rate.[7,36] It is tempting to speculate that lipoproteins may selectively promote the activation of factor IX by factor VIIa at low tissue factor concentrations through the inhibition of the factor X activation pathway. A description of the precise role, if any, that lipoproteins play in the regulation of extrinsic coagulation will obviously require several additional studies.

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CONCISE REPORT

Serum Transferrin Receptor as a New Index of Erythropoiesis

By Yutaka Kohgo, Yoshiro Niitsu, Hitoshi Kondo, Junji Kato, Nobuyasu Tsushima, Katsunori Sasaki, Michiaki Hirayama, Takaaki Numata, Takuji Nishisato, and Ichiro Urushizaki

Serum transferrin receptors were measured by a sandwich radioimmunoassay procedure in patients with iron deficiency anemia, autoimmune hemolytic anemia and aplastic anemia. The mean circulating transferrin receptor concentration of normal subjects and patients with iron deficiency anemia, autoimmune hemolytic anemia and aplastic anemia are 253 ± 82 ng/mL, 730 ± 391 ng/mL, 1,426 ± 1,079 ng/mL, and 182 ± 39 ng/mL, respectively. The values for those with iron deficiency anemia and autoimmune hemolytic anemia were significantly higher than that of normal controls and the values for those with aplastic anemia were lower than that of normal controls. After iron supplementation in iron deficiency anemia, the serum transferrin receptor values increased twofold over those of pretreatment values. This increase parallels an increase in peripheral reticulocytes. Therefore, the number of circulating transferrin receptors in anemic patients may reflect the level of bone marrow erythropoiesis and is a potentially useful new index for red cell production.

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TRANSFERRIN receptor is a transmembrane glycoprotein that is expressed primarily on the surface of erythroid cells and delivers iron during proliferation and hemoglobin synthesis.[1,2] The receptor binds serum diferric transferrin and is endocytosed and transferred to acidosomes, in which inorganic iron is released into the cytosol and the remaining ligand and receptor is recycled back to the cell surface.[3] During this unique recycling process, transferrin receptor was found to be externalized as a form of multivesicular endosomes.[4,5] This externalization of transferrin receptors from the cell surface was first reported by Pan and Johnstone[4] using cultured sheep reticulocytes in vitro. The released receptors may potentially circulate in the serum. Recently, we established a quantitative method for measuring immunoreactive transferrin receptors by using two different monoclonal antibodies, OKT9 and B3/25[6] and found that immunoreactive transferrin receptor was detected at concentrations between 150 and 250 ng/mL. However, the clinical and pathophysiological significance of this circulating transferrin receptor is still obscure. In the present report, we evaluate the usefulness of circulating transferrin receptor as an index of erythropoiesis by measuring its serum level in patients with iron deficiency anemia, autoimmune hemolytic anemia, and aplastic anemia.

MATERIALS AND METHODS

Serum samples. Venous blood samples were taken from 38 normal adult males (age 18 to 72), 54 normal adult females (age 15 to 78), 41 patients with iron deficiency anemia, five patients with autoimmune hemolytic anemia, and five patients with aplastic anemia. The samples were collected before any treatments were started. Serial blood sampling was performed in ten patients with iron deficiency anemia and five patients with autoimmune hemolytic anemia for consecutive determination of serum transferrin receptor values. All serum samples were stored at −20°C until use.

Sandwich radioimmunoassay. Quantitation of immunoreactive serum transferrin receptors by a sandwich radioimmunoassay was performed as previously described.[6] Two monoclonal antibodies against the transferrin receptor, OKT9 and B3/25 were purchased from Ortho Diagnostic (Raritan, NJ) and Hybritech Inc (San Diego), respectively. Flat-bottomed polystyrene plates (Dynatech Lab, Alexandria, VA) were inoculated with 50 μL of OKT9 (10 μg/mL) overnight at 4°C. They were then washed and incubated for one hour at room temperature with 100 μL of phosphate buffered saline containing 1% bovine serum albumin (BSA, Sigma Chemical Co, St Louis) to prevent nonspecific binding. To each well in the plates, 40 μL of purified standard receptor (4 to 2,000 ng/mL) or serum samples was added. The standard transferrin receptors were obtained from human term placenta, which we reported previously.[5] The plates were then incubated another 18 hours at 4°C, washed, and overlayed with 40 μL of ^{125}I-labeled B3/25 (5×10^6 cpm/μg/0.5 mL). After additional incubation for 12 hours at 4°C, the plates were washed and the radioactivity of each well was determined. All samples were performed in triplicate. Radioiodination of proteins was carried out by the lactoperoxidase and glucose oxidase method using Enzymobeads (Bio-Rad, Richmond, VA).[7]

Assay for reticulocyte counts, hemoglobin concentration, serum iron, total iron binding capacity, and serum ferritin. Peripheral reticulocyte counts in 1,000 RBCs were determined by the method of Pappenheim.[8] Hemoglobin was measured by cyanomethemoglobin method.[9] Serum iron and total iron binding capacity (TIBC) were determined by the method of International Committee for Standardization in Haematology.[10,11] Serum ferritin was quantitated by SPAC ferritin kit (Daiichi Radioisotope Lab, Tokyo).[12]

Statistical analysis. Student's *t* test was used to assess the statistical significance of the results shown in the designated tables.

RESULTS

Serum transferrin receptor levels in normal subjects and patients with various anemias are shown in Table 1. The mean values for healthy males and females were 251 ± 94 ng/mL (mean ± 1 SD) (n = 38) and 256 ± 99 ng/mL (n = 54), respectively. No significant differences because of sex or age were observed. The mean values for nontreated patients with iron deficiency anemia and autoimmune hemolytic anemia were 730 ± 391 ng/mL and 1,426 ± 1,079 ng/mL, respectively, both of which were significantly higher than

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Table 1. Serum Transferrin Receptor Levels in Normal Subjects and Patients With Iron Deficiency Anemia, Autoimmune Hemolytic Anemia, and Aplastic Anemia

Diagnosis	No. of Cases	Serum Transferrin Receptor (ng/mL)*	Range	*P* Value†
Normal subjects	92	253 ± 82	70-440	—
Male	38	251 ± 94	80-420	—
Female	54	256 ± 99	70-440	—
Iron deficiency anemia				
Before treatment	41	730 ± 391	100-1,700	<.001
During treatment	14	1,016 ± 326	460-1,800	<.001
After tretment	7	350 ± 147	160-520	NS
Autoimmune hemolytic anemia				
Before treatment	5	1,426 ± 1,079	400-3,010	<.05
After treatment	5	538 ± 150	380-750	<.01
Aplastic anemia	5	182 ± 39	140-240	<.02

In hemolytic anemia, the same five patients were studied before and after treatment with prednisolone.

*Data are expressed as mean ± 1 SD.

†Statistically significant difference from normal values by Student's *t* test.

those of normal subjects. On the other hand, the mean concentration for patients with aplastic anemia was 182 ± 39 ng/mL, which was significantly lower than that of normal subjects. During the iron supplementation in patients with iron deficiency anemia, the concentration increased up to 1,016 ± 326 ng/mL, while the absolute reticulocyte count increased from 2.42 ± 0.97 × $10^4/\mu L$ to 7.30 ± 4.09 × $10^4/\mu L$. Therefore, it seemed that the increase of serum transferrin receptor correlated well with the increase in reticulocytes. After the hemoglobin values were normalized by successful treatment with iron supplementation, the mean value returned to the normal range (350 ± 147 ng/mL) in association with the decreased reticulocyte counts (5.16 ± 1.61 × $10^4/\mu L$). In patients with autoimmune hemolytic anemia, the significant decrease of serum transferrin receptor was observed after effective therapy of prednisolone. The absolute reticulocyte counts before and after steroid therapy were 36.1 ± 6.4 × $10^4/\mu L$ and 15.7 ± 5.8 × $10^4/\mu L$, respectively, indicating that the decline of transferrin receptor in autoimmune hemolytic anemia paralleled the decrease of reticulocyte counts and the improvement of hemoglobin concentration.

Table 2 demonstrates the correlation between serum transferrin receptor values and other hematological parameters such as reticulocyte counts, hemoglobin concentration, TIBC, serum iron, and serum ferritin in patients with iron deficiency anemia and hemolytic anemia. There was a good correlation between serum transferrin receptor and reticulocyte counts in both conditions, suggesting that the increased serum transferrin receptor values may reflect the increase of reticulocytes and bone marrow erythroid cells. In patients with iron deficiency anemia, significant positive or negative correlations were observed between serum transferrin receptor and other markers such as TIBC, serum iron, and serum ferritin concentration, whereas no other correlations were observed in autoimmune hemolytic anemia.

The serial change of serum transferrin receptor values in a patient with iron deficiency anemia supplemented with intravenous (IV) iron administration and in a patient with autoimmune hemolytic anemia treated with prednisolone is shown in Fig. 1. Just after the initiation of iron supplementation, a transient increase of serum transferrin receptors was obtained, and thereafter the values increased again in association with increased reticulocyte counts and returned to normal levels with the improvement of anemia (Fig 1A). In a patient with hemolytic anemia, the serum transferrin receptor level also seems to parallel reticulocyte counts (Fig 1B).

DISCUSSION

We previously reported that immunoreactive transferrin receptor could be detected in human serum by a sandwich radioimmunoassay technique, although no clinical significance was elucidated.[6] In the present report, we measured the receptor concentration in various anemias and obtained the higher values in iron deficiency anemia and autoimmune

Table 2. Correlation Coefficient Between Serum Transferrin Receptor and Other Parameters in Patients With Iron Deficiency Anemia and Autoimmune Hemolytic Anemia

	Iron Deficiency Anemia			Autoimmune Hemolytic Anemia		
Parameters	No. of Cases (n)	Correlation Coefficient (r)*	*P* Value†	No. of Cases (n)	Correlation Coefficient (r)*	*P* value†
Reticulocyte counts	36	0.688	<.01	5	0.755	<.05
Hemoglobin concentration	36	−0.200	NS	5	−0.446	NS
Total iron binding capacity	10	0.593	<.05	5	−0.217	NS
Serum iron	10	−0.651	<.05	5	0.347	NS
Serum ferritin	10	−0.703	<.05	5	0.460	NS

*Correlation between serum transferrin receptor and various parameters.

†Statistically significant linear regression.

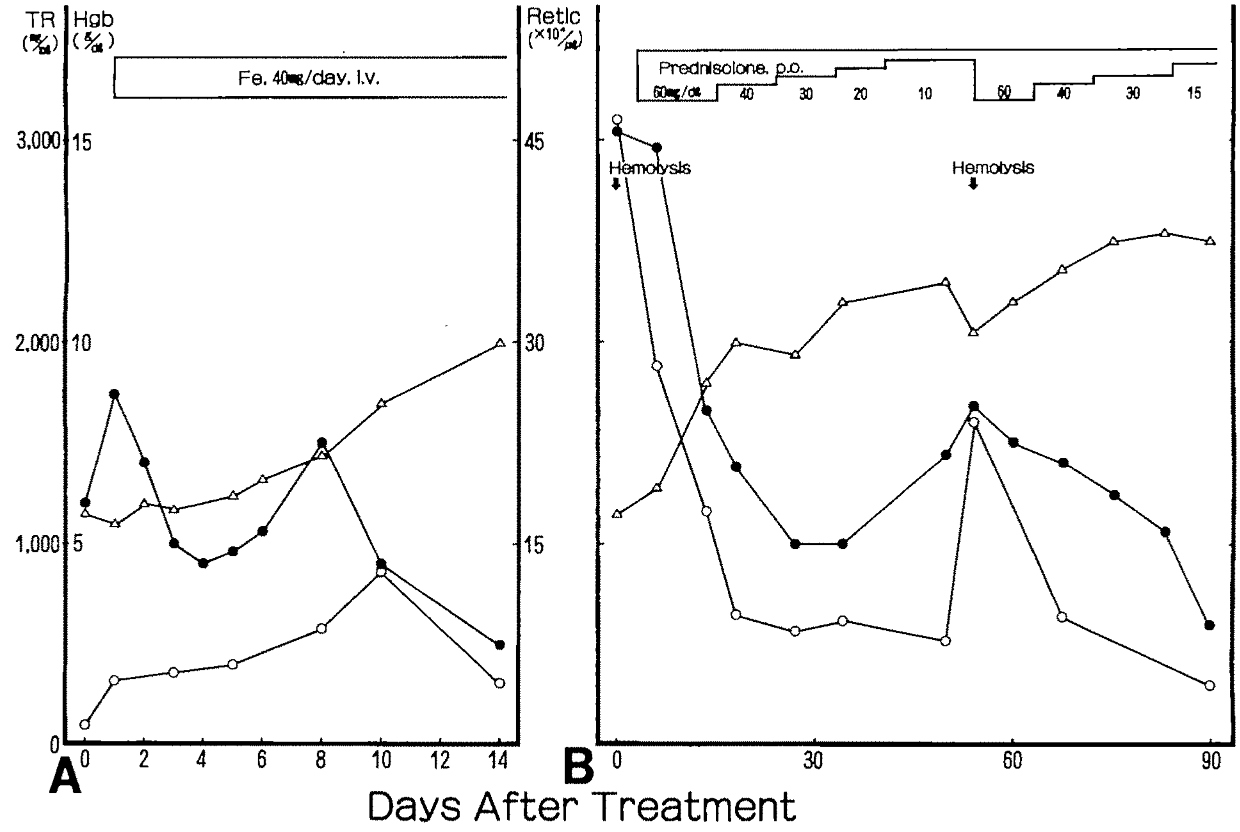

Fig 1. Changes of serum transferrin receptor (TR), hemoglobin concentration (Hgb) and peripheral reticulocyte counts (Retic). (A) A patient with iron deficiency anemia during iron supplementation. (B) A patient with autoimmune hemolytic anemia during treatment with prednisolone. TR, ●; Hgb, △; Retic, ○.

hemolytic anemia and lower values in aplastic anemia (Table 1). These values paralleled the peripheral reticulocyte counts (Table 2), suggesting that the serum transferrin receptors may reflect the turnover of transferrin receptors in erythrocyte progenitor cells in the bone marrow.

Since Jandl and Katz[13] first reported the presence of transferrin receptors on the reticulocyte surface, erythrocyte progenitor cells have been considered the main source of transferrin receptors in the body as well as syntrophoblasts of placenta and malignant tissues.[1] The overall dynamics of transferrin receptors has been extensively studied using the erythroleukemia cell line, K562[3], and the expression of transferrin receptors is thought to be regulated by the equilibrium between its recycling and synthesis. Receptor mRNA synthesis is increased with the addition of iron deficient serum and iron chelating agents in vitro.[14] Therefore, it is likely that the reticulocytes in iron deficiency anemia may possess more receptors than usual.

Good correlations between serum transferrin receptor value and markers such as TIBC, serum iron, and serum ferritin were present only in iron deficiency anemia but not in autoimmune hemolytic anemia (Table 2). This result may explain that there is a double effect in iron deficiency anemia, ie, iron deficiency and erythropoiesis. Actually, in autoimmune hemolytic anemia, which could exclude the effect of iron deficiency, there is no correlation with TIBC, serum iron or serum ferritin.

According to Pan and Johnstone,[4] the receptors of reticulocytes were shed and released into the medium. We also found the same phenomenon in K562 cells.[5] The shedding of transferrin receptor was inhibited by metabolic inhibitors such as cytochalasin B, colchicine, and sodium azide. The presence of ligand, on the other hand, increased this externalization. These results suggest that this externalization of receptor is a ligand-dependent and energy-dependent process. Therefore the measurement of shed circulating receptors in the serum seems to be a practical and efficient way for estimating erythrocyte transferrin receptor turnover. The lower levels in aplastic anemia reflect decreased erythropoiesis in the bone marrow. Interestingly, the biphasic increase of serum transferrin receptor levels was obtained in the clinical course of iron deficiency anemia during which IV iron was supplemented (Fig 1A). The transient increase was observed just after iron supplementation and the second increase was observed two to three days before the increase in reticulocytes. The first increase, just after iron supplementation, may be explained by the transient increase of receptor shedding according to the increase of serum diferric transferrin, which facilitates the internalization and recycling-shedding process of the receptors. On the other hand, the second increase paralleled the reticulocyte increase may reflect the increase of erythron, namely the accelerated division and proliferation of erythroid precursor cells by incorporation of iron into the cells. It is noteworthy that serum transferrin receptor appeared two to three days before the increase in reticulocytes. In patients with autoimmune hemolytic anemia, the serum transferrin receptor level also seems to parallel reticulocyte counts (Fig 1B) and the serial determination was useful for monitoring the activity of the bone marrow erythroid mass. Therefore, the measurement of serum transferrin receptor is a simple and rapid method for estimation of erythropoiesis, formerly estimated by reticulocyte counts or complicated ferrokinetic measurements such as plasma iron turnover (PIT) and percent red cell use (%RCU).[15] In conclusion, the measurement of circulating transferrin receptor in patients with various anemias may be a useful new index for estimating the bone marrow erythropoiesis.

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CONCISE REPORT

Coordinate Glycosylation and Cell Surface Expression of Glycophorin A During Normal Human Erythropoiesis

By Michael R. Loken, Curt I. Civin, William L. Bigbee, Richard G. Langlois, and Ronald H. Jensen

The expression of two epitopes on glycophorin A (GPA) during erythroid development was examined on normal human bone marrow using quantitative flow cytometry. The highly correlated binding of two monoclonal antibodies, one sensitive and the other insensitive to glycosylation, indicated that the two epitopes were coordinately expressed during erythroid development. Both antigens reached a maximum expression during the early normoblast stage and were maintained at a constant amount per cell throughout further maturation to erythrocytes. These data suggest that glycosylation of GPA, as detected by antibodies recognizing blood group (M) and (N) antigens, does not increase during erythroid maturation.

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GLYCOPHORIN A (GPA) is the major sialoglycoprotein of the human erythrocyte membrane.[1,2] This protein is restricted to the erythroid lineage.[3] Cell surface expression of GPA is first detected at the erythroblast stage of development, after the cells can no longer form erythroid colonies.[3,4] GPA occurs in two allelic forms corresponding to the (M) and (N) antigens of the MN blood group.[5,6] The (M) and (N) antigens are identical except for two amino acid differences in the amino terminal region, but 0-linked tetrasaccharides in this region are required for the binding of many (M) and (N) specific antibodies.[7-9]

Biochemical analyses of cell lines (K562 and HEL) stimulated to undergo maturation and comparisons of abnormal with normal bone marrow suggested that GPA acquired an increased number of 0-linked tetrasaccharides when the erythroid cells matured.[10] These conclusions were supported by experiments using antibodies that detected (M) and (N) blood group antigens.[11]

In studying the expression of GPA during normal erythropoiesis, we showed that GPA reached maximal expression at the basophilic normoblast stage and remained in a constant amount per cell throughout further maturation to the erythrocyte.[4] The reported late glycosylation of GPA during erythrocyte development was difficult to explain in light of this invariance of amount of GPA on the maturing erythroid cells. Therefore, we investigated the correlated expression of two epitopes of GPA during erythropoiesis using monoclonal antibodies, one epitope was insensitive to glycosylation while the other required intact tetrasaccharides.

MATERIALS AND METHODS

Monoclonal antibodies. Anti-GPA (M) (9A3) binds only to type (M) glycophorin A while anti-GPA (M/N) (10F7) identifies both types (M) and (N) GPA.[12-14] Fluorescein isothiocyanate (FITC) was used to prepare directly conjugated 10F7.[15] Rat anti-mouse kappa phycoerythrin (PE) and isotype control IgG_1 FITC was obtained from Becton Dickinson Immunocytometry Systems, Mountain View, CA.[16]

Cell preparation and immunofluorescence. Bone marrow aspirates were obtained from three consenting normal adult volunteers. Collection of samples was performed after approval by the Institutional Review Board, as approved by the Department of Health and Human Services. The low-density leukocytes (1.077 g/mL) were stained using indirect immunofluorescent as described.[4] Briefly, 10^6 cells were incubated sequentially with anti-GPA(M), rat anti-mouse kappa PE, 10% normal mouse serum, and anti-GPA(M/N) FITC with a wash interspersed between each step. As a negative control an IgG_1 FITC monoclonal antibody with irrelevant specificity was used in place of the anti-GPA (M/N). The cells were fixed in 1% paraformaldehyde for later analysis.[17] Some bone marrow cells were incubated with 0.5 IU neuraminidase for one hour at 37°C (Sigma, St Louis) before immunofluorescence staining.[12] The data from three separate experiments gave identical results.

Flow cytometry. Quantitative fluorescence analyses were performed on a FACS 440 (Becton Dickinson) using the filter combinations previously described.[18] Linear amplifiers were used for the light scattering signals, while 4-decade logarithmic amplifiers were used for the fluorescence signals. A minimum of 20,000 events were collected in list mode with contour levels drawn at 2, 4, 8, 16, 32, 64, and 128.

RESULTS

All immunofluorescence analyses were performed by gating on either the "Lymph" or the "Blast" light scattering populations identified for normal bone marrow (Fig 1). No staining above background was found for either the anti-GPA (M) specific or the anti-GPA (M/N) binding antibody on cells which were in the "Gran" light scattering window (data not shown).

The indirect immunofluorescence staining with the anti-GPA (M) specific antibody used rat anti-mouse kappa PE as a second step. A distinct population of labeled cells was observed in both the "Lymph" and "Blast" light scattering windows (Fig 2A). The lack of staining with the negative control, IgG_1 FITC, indicated that there was complete blocking of the free valencies of the rat anti-mouse kappa PE by normal mouse serum used in the staining protocol.

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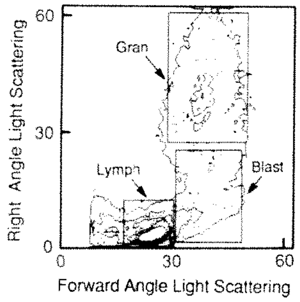

Fig 1. Correlation of forward and right angle light scattering of low density human bone marrow after immunofluorescence staining and fixation. Three populations of cells are identifiable in this plot:"Lymph" including lymphocytes, mature erythrocytes, and late normoblasts; "Blast" including early normoblasts, erythroblasts, monocytes, monoblasts, and myeloblasts; "Gran" exclusively neutrophil lineage from promyelocyte to mature neutrophil.[4] The fluorescence data in Fig 2 were gated on either the "Lymph" or "Blast" light scattering window. The differences observed between this light scattering pattern and that previously reported are a consequence of the fixation of the cells.[4]

When the anti-GPA (M) and anti-GPA (M/N) antibodies were used together to stain the sample, they bound to the same cells (Fig 2B). There were no cells that stained with the anti-GPA (M) (9A3) antibody that did not also bind the anti-GPA (M/N) (10F7) antibody and vice versa. In addition, there was a tight quantitative correlation of binding of the two antibodies on the cells (Fig 2B, "Blast"). The intensity of fluorescence of the anti-GPA (M) staining was the same whether or not the anti-GPA (M/N) was bound to the cells. These data suggest that the two antibodies identified the same antigen but bound at different epitopes[19] since they did not inhibit the attachment of the other antibody.

An aliquot of the same bone marrow preparation was treated with neuraminidase (which selectively removes sialic acid residues) before immunofluorescence labeling by the two anti-GPA antibodies. Binding of anti-GPA (M) was lost with the removal of the terminal sialic acid residues while no difference in intensity of anti-GPA (M/N) binding (Fig 2C). This illustrated the absolute requirement of the terminal sialic acids for the binding of the (M) specific antibody. This enzymatic treatment had no effect on the binding of the anti GPA (M/N) antibody.

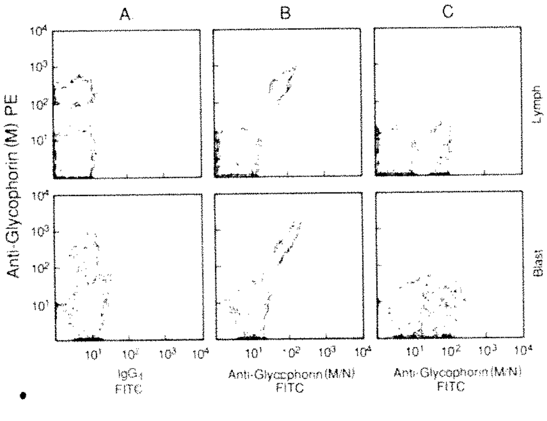

Fig 2. Correlation between anti-GPA (M) specific and anti-GPA (M/N) reactive monoclonal antibodies on normal human bone marrow. (A) Comparison of anti-GPA (M) specific antibody with a negative control of IgG, FITC. (B) Fluorescence analysis of marrow cells double labeled with anti-GPA (M) and anti-GPA (M/N) antibodies. (C) Fluorescence analysis of marrow cells treated with neuraminidase before labeling with both anti-GPA (M) and anti-GPA (M/N) antibodies.

DISCUSSION

Both of the anti-GPA monoclonal antibodies used in this study recognize the amino terminal peptide of the glycophorin A molecule with essentially no reactivity with glycophorin B.[12] The anti-GPA (M) specific antibody (9A3) requires the terminal sialic acid residues for binding, as shown previously on erythrocytes[12] and, in this study, on less mature erythroid lineage cells (Fig 2C). The binding of the anti-GPA (M/N) antibody (10F7) is unaffected by neuraminidase treatment, both on mature erythrocytes and on immature erythroid cells (Fig 2C).[12] Anti-GPA (M/N) also reacts with cells which lack all but the first carbohydrate residue of the tetrasaccharides on the GPA.[13] These data suggest that the anti-GPA (M/N) probably binds to a glycosylation insensitive site somewhat removed from the *N*-terminus.[12]

The highly correlated expression of the two epitopes shown in Fig 2B suggests that the two antibodies identify not only the same populations but that the two epitopes were coordinately expressed on those cells. Since all maturational stages of erythroid development are represented in the normal bone marrow, these data indicate that the two epitopes appear at the same time during erythroid maturation. Thus, GPA is sialated at the time that it is first expressed on the cell surface.

The morphological analysis of cells sorted on the basis of GPA intensity indicated that GPA first appears during the erythroblast stage.[4] Cells with intermediate amounts of GPA were >90% erythroblasts while those expressing maximal amounts of GPA were predominantly normoblasts. The less mature normoblasts were larger than the more mature cells and could be distinguished by light scattering differences. Since both the anti-GPA (M/N) and anti-GPA (M) epitopes follow the same pattern of expression during maturation, it can be concluded that there is no change in the sialic acid residues on these epitopes on GPA after the erythroblast stage. The anti-GPA (M/N) and the anti-GPA (M) epitopes both reach a maximum amount per cell and remain constant during further maturation of the cells to erythrocytes.

If there were glycosylation during the later normoblast stages of maturation, a discordance in immunofluorescence staining would be observed, as has been demonstrated with other erythroid cell surface antigens such as transferrin receptor and CD45 (common leukocyte antigen).[4]

The observations by Ekblom et al may have resulted from less precise quantitation of the binding of their monoclonal antibodies and their rabbit antiserum.[10,11] In addition, the flow cytometric experiments detected only cell surface antigens while immunofluorescence staining after fixation on a slide identified both cell surface and cytoplasmic antigens.[11]

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CONCISE REPORT

tdic(9;12): A Nonrandom Chromosome Abnormality in Childhood B-Cell Precursor Acute Lymphoblastic Leukemia: A Pediatric Oncology Group Study

By Andrew J. Carroll, Susana C. Raimondi, Dorothy L. Williams, Frederick G. Behm, Michael Borowitz, Robert P. Castleberry, Michael B. Harris, Richard B. Patterson, D. Jeanette Pullen, and William M. Crist

In a review of 432 children with newly diagnosed acute lymphoblastic leukemia (ALL), we identified a new nonrandom translocation, tdic(9;12)(p1?1;p1?2), in the leukemic marrow cells of eight patients. Seven had hypodiploid karyotypes that lacked chromosomes 9 and 12 and contained a der(12), tdic(9;12); the eighth had a pseudodiploid karyotype with two normal 9 chromosomes, one normal 12 and the der(12), tdic(9;12). Abnormalities involving chromosomes other than 9 and 12 were noted in four of the eight patients. All cells with the tdic(9;12) expressed both the common ALL antigen and HLA-DR. Cytoplasmic immunoglobulin, a marker of pre-B ALL, was detected in one case with the tdic(9;12) but was absent in the other seven. Our results suggest that the tdic(9;12)(p1?1;p1?2) rearrangement is specifically associated with leukemic B cell precursors.

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CHROMOSOMAL rearrangements have been identified as the single most important prognostic variable in childhood acute lymphoblastic leukemia (ALL).[1] Moreover, certain nonrandom rearrangements, including the t(4;11) in infants with undifferentiated acute leukemia, the t(8;14) in B cell ALL, the t(1;19) in pre-B cell ALL,[2,3] and the t(11;14) and t(10;14) in T cell ALL,[3,4] are associated with specific clinically and immunologically defined subclasses of ALL. Other chromosomal abnormalities often found in the blast cells of patients with ALL include the t(9;22),[5] 6q−[5] and a deletion or rearrangement of 9p[6-9] and of 12p.[10,11] These structural abnormalities are usually found in pseudodiploid karyotypes and are seen less frequently in other ploidy groups. We have reviewed the cytogenetic features of 432 children with newly diagnosed ALL and have found a new nonrandom translocation, tdic(9;12)(p1?1;p1?2), in the leukemic B cell precursors of eight patients, seven of whom had hypodiploid karyotypes.

MATERIALS AND METHODS

Patients. A total of 432 consecutive cases of ALL with fully banded chromosomes were available for review. Their distribution according to referral center was: St Jude Children's Research Hospital, 187 cases studied between 1984 and 1986; University of Alabama at Birmingham (UAB), 100 cases studied between 1979 and 1985; and the Pediatric Oncology Group (POG) 8600 study, 145 cases in which marrow specimens were shipped to the UAB cytogenetics laboratory for chromosome analysis between January and June 1986. All patients were advised of the procedures and attendant risks, in accordance with institutional guidelines, and informed consent was obtained in each instance.

Immunologic characterization. After separation on a Ficoll-Hypaque gradient, the leukemic marrow cells were evaluated by standard techniques for surface immunoglobulins (sIg),[12] cytoplasmic immunoglobulins (cIg),[13] and receptors for sheep erythrocytes.[14] In cases of pre-B cell or B cell ALL, >10% of the blasts expressed either cIg or sIg. A case was considered E-rosette positive if >40% of the blasts formed heat stable E-rosettes. Surface antigens, including CALLA (CD10), B1 (CD20), and HLA-DR, were determined by indirect immunofluorescence or, in some cases, by microcytotoxicity assays with use of heteroantisera, as previously described.[15] In the cytotoxicity assays, 20% reactivity above control was considered positive in immunofluorescence.

Chromosome analysis. Bone marrow samples from the 187 patients studied at St Jude Hospital were processed directly according to the method of Williams et al.[16] Samples from the 100 patients seen at UAB were either processed directly or placed in short-term culture, or both.[9] Samples from the 145 POG patients were placed in RPMI 1640 supplemented with 15% fetal calf serum and shipped overnight to the UAB cytogenetics laboratory. On arrival, they were subjected to short-term (24 hour) culture.[9] Routine methods were used for culture harvest, slide preparation, and GTG-banding. Karyotypic designations were assigned according to the International System for Human Cytogenetic Nomenclature.[17]

RESULTS

Three hundred twenty-eight (76%) of the 432 cases studied had a cytogenetically abnormal clone, eight of which (Table 1) showed a translocation involving chromosomes 9 and 12. The resulting derivative chromosome appeared to be dicentric with breakpoints tentatively assigned at 9p11 and 12p12; consequently, the rearrangement was designated tdic(9;12)(p1?1;p1?2)(Fig 1). We are aware that, on close examination of the derivative chromosomes shown in Fig 1, it could be reasonably suggested that the chromosome breakpoints are slightly different in each of the three cases. While we cannot rule out case to case variation in breakpoints, it is also possible that the apparent differences are the result of contracted chromosomes since we observed a similar degree

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Table 1. Cytogenetic Findings in the Eight Cases With tdic (9;12)

Case No.	No. of Metaphases Examined	Abnormal Metaphases (%)	Abnormal Karyotype
1	21	20	45,XY,−9,−12,+der(12),tdic(9;12)(p11;p12)
2	22	45	45,XY,−9,−12,+der(12),tdic(9;12)(p11;p12)
3	21	90	45,XY,−9,−12,+der(12),tdic(9;12)(p11;p12)
4	20	80	45,XY,−9,−12,+der(12),tdic(9;12)(p11;p12)
5	17	50	45,XY,del(X)(q26),−9,−12,+der(12),tdic(9;12)(p11;p12)
6	21	95	45,XY,del(7)(p15),−9,−12,+der(12),tdic(9;12)(p11;p12)
7	20	80	45,XX,−5,−9,−12,+der(12),tdic(9;12)(p11;p12),+mar(?14),?dup(6)(q21q26)
8	20	75	46,XY,−12,+der(12),tdic(9;12)(p11;p12)(60%)/45,XY,−8,−9,−12+der(8),t(8;9)(p23;q13),+der(12),tdic(9;12)(p11;p12)(15%)

of variation between metaphases from individual patient cultures. The fact that the alternate derivative chromosome, containing the remaining short-arm material from chromosomes 9 and 12, was absent in all eight cases supports our contention that the translocation results in a dicentric chromosome. Furthermore, C-banding demonstrated the presence of the 9qh region on the der(12) chromosome in each of the three cases examined in this manner. Seven cases were hypodiploid, lacking a 9 and a 12 chromosome, and contained a der(12),tdic(9;12). The eighth case had a pseudodiploid karyotype with two normal 9 chromosomes, one normal 12 and the der(12),tdic(9;12). Abnormalities involving chromosomes other than 9 and 12 were noted in four of the eight cases. One case had two cytogenetically abnormal clones, both with the tdic(9;12). The proportions of cytogenetically normal cells in each case ranged from 5% to 80%.

Table 2 lists the clinical and laboratory findings in the eight cases with the tdic(9;12). Blast cells uniformly expressed both CALLA and HLA-DR. In five of seven cases in which it was determined, blast cells also expressed CD20 (B1). In seven cases the blast cells were morphologically classified as FAB L1. Cytoplasmic Ig was detected in one case. With the possible exception of male predominance (M:F ratio, 7:1), there were no obvious variations in clinical features that would distinguish these patients from the larger group with B cell precursor ALL lacking the tdic(9;12). Leukocyte counts ranged from 2.9 to 132 × 10^9/L and ages from 3 to 16 years. Three patients had extramedullary disease at the time of the diagnosis.

DISCUSSION

These results suggest that a rearrangement involving the short arms of chromosomes 9 and 12, tdic(9;12)(p1?1;p1?2) is an acquired, nonrandom chromosome abnormality with a frequency of ~2% among children with ALL. It appears to be limited to cases of B cell precursor ALL, specifically those

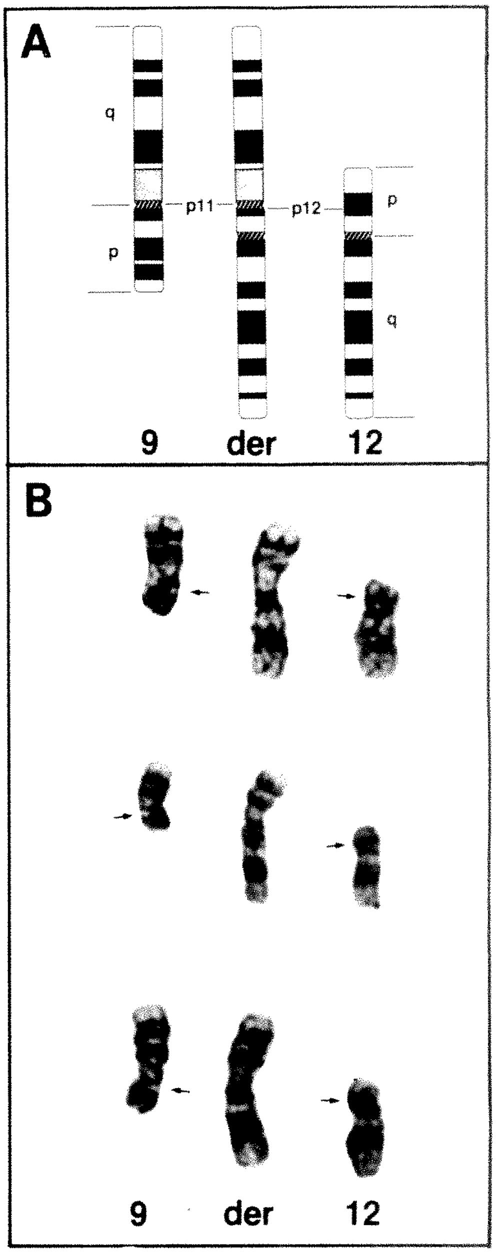

Fig 1. The tdic(9;12)(p1?1;p1?2) associated with B cell precursor ALL. (A) Diagram showing the G-banded pattern of the derivative (der) chromosome and normal homologues involved in the tdic(9;12). (B) Representative partial karyotypes from three different patients with the tdic(9;12). Top, case no. 1; middle, case no. 6; and bottom, case no. 8. The arrows indicate the approximate positions of the breakpoints.

characterized by CALLA, HLA-DR, and usually CD20 expression, a phenotype seen in 25% to 30% of cases of ALL in children. It may occur much more frequently in boys, as has been noted for chromosome 9p abnormalities in ALL.[6,7,9]

We are aware of only two other reports of a similar 9;12 translocation. The LAZ 221 cell line, established from the peripheral blood of a patient with "null" cell ALL, was characterized as having a karyotype of 45,XX,−9,−12,

Table 2. Clinical and Laboratory Findings for the Eight Cases With tdic(9;12)

Case No.	FAB Type	Age(yr)	Sex	WBC (× 10/L)	Hb (mg/dL)	Immunophenotype	Cell Markers CALLA	HLA-DR	B1	Extramedullary Disease
1	L1	3	M	2.9	4.1	Common	+	+	+	−
2	L1	16	M	6.1	12.3	Common	+	+	+	−
3	L1	15	M	19.0	11.2	Common	+	+	+	+
4	L1	4	M	15.1	8.7	Common	+	+	−	−
5	L1	4	M	132.0	4.4	Common	+	+	−	+
6	L1	8	M	44.2	6.5	Common	+	+	NT	−
7	L2	6	F	8.2	5.8	Common	+	+	+	−
8	L1	12	M	3.7	9.2	Pre-B	+	+	+	+

Abbreviation: NT, not tested.

+t(9q12q).[18] In a review of 70 cases of ALL, Heerema et al[19] identified a child whose blast cell karyotype was 45,XY,−9,−12,+der(12),t(9;12)(q1;p13). Further examination of this case indicated that the abnormality was the same as that in our eight cases (N.A. Heerema, personal communication, January 1987).

Deletions or rearrangements involving the short arm of chromosome 9 or the short arm of chromosome 12 seem to be equally common among children with ALL, with each occurring in ~10% newly diagnosed cases.[6-11] Thus, the tdic(9;12) may define a distinct subgroup of patients who could be included in either or both of these cytogenetic categories. In fact, cases no. 2, 5, and 6 were previously included in reports describing 9p or 12p abnormalities.[8-11] In addition, cases no. 3 through 5 were included in a report[20] concerning hypodiploidy and its association with treatment outcome in childhood ALL.

Many nonrandom chromosomal translocations associated with chronic or acute leukemia involve breakpoints at or near the assigned loci for genes encoding proteins that affect cell growth. In view of the fact that the c-Kirsten-*ras* 2 oncogene has been mapped to 12p11.1-p12.1,[21] molecular studies of cases with the tdic(9;12) appear warranted.

The clinical significance of the tdic(9;12) is uncertain since so few patients with the abnormality have been reported and the follow-up times for most of them are short. Nevertheless, all eight patients described in this report attained complete remission and remain free of leukemia for 5 to 36 months (median, 10 months). Previous reports indicating that the presence of a chromosomal translocation[1] or hypodiploidy[20] confers a poor prognosis suggest that patients with the tdic(9;12) are at increased risk for treatment failure; however, longer follow-up is required to substantiate this impression.

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CONCISE REPORT

Host Origin of the Human Hematopoietic Microenvironment Following Allogeneic Bone Marrow Transplantation

By Joseph Laver, Suresh C. Jhanwar, Richard J. O'Reilly, and Hugo Castro-Malaspina

The origin of marrow stromal cells post allogeneic bone marrow transplantation (BMT) was studied. Two groups of patients receiving HLA-identical marrow grafts from sex mismatched siblings were included in the study: the first group (eight patients) received conventional marrow grafts and the second group (ten patients) received stromal cell and T cell depleted grafts. All patients showed hematopoietic engraftment with donor cells. Marrow aspirates obtained from these patients were used to establish stromal layers in long-term marrow cultures (LTMC) for 4 to 6 weeks. In both groups, karyotype analysis of nonhematopoietic cultured stromal cells showed host origin even as late as day 760 posttransplantation. Immunofluorescence methods using monoclonal antibodies against components of fibroblasts, macrophages, and endothelial cells, showed that the composition of stromal layers was similar to those obtained from normal controls. Our data indicate that marrow stromal progenitors capable of proliferation are nontransplantable and do not originate from a hematopoietic-stromal common progenitor.

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AN INTACT hematopoietic microenvironment is required for successful engraftment of donor hematopoietic stem cells.[1] The origin (host *v* donor) of this marrow compartment following bone marrow transplantation (BMT) is still controversial. Keating et al[2] reported that the microenvironment becomes progressively of donor origin, while other studies assessing stromal cell subpopulations suggested a host origin.[3,4] Donor origin would imply that stromal engraftment is required for successful marrow transplantation, whereas host origin would imply that the indigenous stroma remains functionally active following preparatory pretransplant treatment. In case of host origin, the design of new aggressive cytoreductive protocols should consider the effects of such therapy on the marrow microenvironment. Since failure or delay of hematopoietic reconstitution might be due to an impaired marrow microenvironment, it is important to determine the origin of these cells. A better understanding of the origin of marrow stromal cells can be reached by comparative analysis of stromal and hematopoietic reconstitution in recipients of stromal cell-containing and stromal cell-depleted marrows. The latter marrow grafts that are depleted of stromal cells by soybean lectin agglutination,[5] provide a unique model for studying the transplantability and the origin of stromal cells following BMT. In this study, we have addressed these questions by using chromosomal analysis of cultured marrow microenvironmental cells derived from patients who had received either conventional or stromal depleted marrow grafts from HLA-identical donors, and showed host origin in all instances.

MATERIALS AND METHODS

Patients. Eighteen patients who received allogeneic marrow grafts from sex mismatched HLA identical siblings were studied. After informed consent was obtained, patients were prepared for transplantation with hyperfractionated total body irradiation and cyclophosphamide (except one patient with severe aplastic anemia who received total lymphoid irradiation and cyclophosphamide).[6] Eight patients received conventional grafts and ten received marrows depleted of stromal progenitors and T cells. The separation method, involving soybean agglutination and E-rosetting (SBA), was originally used to eliminate T cells[7] and was subsequently found to deplete stromal progenitors as well.[5]

Long-term marrow cultures. Bone marrow aspirates post-BMT were obtained at 14 to 760 days in the conventional group and 14 to 563 days in the SBA separated group. Long-term cultures of human marrow (LTMC) were established according to the method of Gartner and Kaplan[8] with some modifications.[9] Briefly, 20×10^6 marrow buffy coat cells in 10 mL of LTMC medium were inoculated into T-25 tissue culture flasks. Cultures were then gassed with 5% CO_2 in air and incubated at 33°C. The LTMC medium consisted of supplemented McCoy's 5A medium containing 12.5% horse serum, 12.5% FCS and 10^{-6} mol/L hydrocortisone sodium succinate (Calbiochem-Behring, La Jolla, CA). At weekly intervals, half the supernatant medium and suspension cells were removed and replaced with fresh LTMC medium. The development of marrow stromal layers was assessed every seven days under a phase contrast inverted microscope and the percentage of the flask surface covered by the stromal interlocking network was visually estimated and recorded.

To evaluate the composition of the adherent stromal cells in culture we used immunofluorescence methods using antibodies to components of fibroblasts (collagen type III and fibronectin), endothelial cells (factor VIII related antigen), and macrophages (monocyte surface molecules recognized by 63D3 and 61D3 mouse monoclonal antibodies), as previously described.[9]

Cytogenetic studies. To assess the origin of hematopoietic and stromal cells, chromosomal analysis of fresh bone marrow cells and trypsinized LTMC stromal cells was performed using quinacrine dihydrochloride staining (Q-banding).[10] The binomial distribution was used to calculate the probability to identify donor stromal cells derived from LTMC.

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Table 1. Karyotype Analysis of Marrow Hematopoietic and Stromal Cells Following Unseparated Grafts

			Sex			Marrow Karyotype*	
UPN	Age (yr)	Diagnosis	Host	Donor	Day Post-BMT	Hematopoietic†	Stromal
1	7	ALL	M	F	33	46,XX(30/30)	46,XY(30/30)
					760	46,XX(30/30)	46,XY(34/34)
2	7	AML	M	F	150	46,XX(18/18)	46,XY(30/30)
3	8	AML	F	M	30	46,XY(30/30)	46,XX(16/16)
					60	46,XY(30/30)	46,XX(32/32)
4	8	ALL	M	F	14	46,XX(30/30)	46,XY(10/10)
					74	46,XX(29/29)	46,XY(32/32)
5	8	AA	M	F	120	46,XX(30/30)	46,XY(28/28)
					640	46,XX(30/30)	46,XY(32/32)
6	11	AML	M	F	101	46,XX(16/16)	46,XY(34/34)
7	12	ALL	M	F	31	46,XX(16/16)	46,XY(9/9)
8	14	AML	F	M	50	46,XY(30/30)	46,XX(24/24)

Abbreviations: AML, acute myelocytic leukemia; AA, aplastic anemia.

*In parentheses appears the number of cells with given karyotype as fraction of total number of cells studied.

†Donor's karyotype.

RESULTS

The eighteen patients included in this study showed engraftment with donor hematopoietic cells. Hematopoietic cells remained of donor origin throughout the study period. In contrast, chromosomal analysis of cultured stromal cells in 4 to 6-week-old LTMC showed host origin in recipients of either unmodified (group 1) or stromal cell depleted marrow grafts (group 2) (Tables 1 and 2). In both groups the composition of stromal layers in LTMC derived from marrows obtained over day 35 of the BMT was similar to that seen in normal controls and included fibroblasts, lipid-containing cells, macrophages, and 5% to 10% endothelial cells (Table 3). In group 1, 311 stromal metaphases were studied. All, including 158 over day 100 post-BMT, showed host origin. In group 2, 367 metaphases including 273 over day 100 post-BMT were studied showing host stromal origin. It is unlikely that the presence of donor stroma was missed as the probability to do so is extremely low. For instance, if the frequency of donor stromal cells was 5%, the probability that none would be identified in a random sample of over 300 metaphases is <0.1%. Furthermore, in our laboratories we have been able to identify as low as 3% mosaicism in marrow specimens obtained after marrow transplantation.

DISCUSSION

The data presented show the host origin of cultured marrow stromal cells obtained from patients successfully transplanted with allogeneic grafts. These findings suggest that chemoradiotherapy used for myeloablation does not fully ablate marrow stromal progenitors. Achievement of successful hematopoietic engraftment in these patients indicates that the host microenvironment plays an important role in the posttransplant period: In the early stages it provides favorable conditions for hematopoietic engraftment, and later it reconstitutes a functional microenvironment capable of sustaining hematopoiesis. Since the reconstitution of the marrow microenvironment requires proliferation of stromal

Table 2. Karyotype Analysis of Marrow Hematopoietic and Stromal Cells Following Stromal Depleted Grafts

			Sex			Marrow Karyotype*	
UPN	Age (yr)	Diagnosis	Host	Donor	Day Post-BMT	Hematopoietic†	Stromal
9	10	ALL	M	F	226	46,XX (30/30)	46,XY (16/16)
10	15	ALL	M	F	35	46,XX (23/24)	46,XY (12/12)
					116	46,XX (28/29)	46,XY (32/32)
11	15	NHL	M	F	28	46,XX (30/30)	46,XY (12/12)
					42	46,XX (30/30)	46,XY (8/8)
12	16	AML	M	F	14	46,XX (27/27)	46,XY (40/40)
					110	46,XX (16/16)	46,XY (30/30)
13	28	CML	F	M	109	46,XY (12/12)	46,XX (12/12)
14	28	CML	F	M	24	46,XY (30/30)	46,XX (10/10)
					103	46,XY (24/24)	46,XX (24/24)
					324	46,XY (30/30)	46,XX (34/34)
15	33	CML	M	F	309	46,XX (30/30)	46,XY (29/29)
16	33	AML	F	M	21	46,XY (29/29)	46,XX (12/12)
					390	46,XY (30/30)	46,XX (30/30)
17	34	AML	M	F	563	46,XX (26/26)	46,XY (32/32)
18	36	CML	F	M	287	46,XY (30/30)	46,XX (34/34)

Abbreviations: NHL, non-Hodgkin lymphoma; CML, chronic myelocytic leukemia.

*In parentheses appear the number of cells with given karyotype as fraction of total number of cells studied.

Table 3. Cellular Composition of Stromal Layers in Long-Term Marrow Culture

	Relative Frequency*	
Cell Type	Stromal Layers Derived From Post-BMT Marrows	Stromal Layers Derived From Normal Marrows
Fibroblast	+ + + +	+ + + +
Adipocyte	+ +	+ +
Endothelial cell	+	+
Macrophage	+ + +	+ + +

The cellular composition was assessed by immunofluorescence staining with antisera against type III collagen and fibronectin for fibroblasts, and against factor VIII-related antigen for endothelial cells, as well as monoclonal antibodies recognizing monocytic antigens and by light microscopy for adipocytes. The presence and relative amount of different cell types were assessed semiquantitatively by grading their frequency from + to + + + +: +5% to 10%; + +, 11% to 25%; + + +, 26% to 50%; + + + +, 50%.

*Cultures were initiated with 20 × 10^6 marrow cells per flask and after six weeks of incubation adherent layers were examined for their cellular composition.

progenitors it is important to determine the origin of stromal cells capable of undergoing mitosis.

Our results showing host origin of proliferating marrow microenvironmental cells following BMT suggest that stromal progenitors are nontransplantable. The apparent discrepancy between our results and those reported by Keating et al[2] can be explained by the differences in the techniques used to assess cultured stromal cells and the type of cells analyzed by these methods. We used chromosomal analysis, which determines the origin of stromal cells capable of undergoing mitosis, whereas Keating by determining the presence of the Y-body chromosome analyzed nondividing cells (cells in interphase). Identifying donor derived macrophages that are abundantly present in stromal layers with the Y-body method could lead to the incorrect conclusion that stromal cells post BMT are of donor origin. In contrast, chromosomal analysis assesses only cells undergoing mitosis and excludes differentiated nondividing cells such as macrophages. Using karyotype analysis, other investigators have also shown that certain stromal elements, such as marrow fibroblasts and reticulofibroblastoid cells, are of host origin following allogeneic unmodified marrow grafts.[3,4] Analogous discrepancy exists regarding the origin of stromal cells in patients with clonal hematopoietic disorders such as chronic myelocytic leukemia. This, once again, can be explained by the different cell populations used for study (dividing *v* nondividing cells). Chromosomal analysis has demonstrated that stromal cells do not originate in the malignant clone[3,11] whereas G6PD studies[12] of nondividing cells in patients heterozygous for types A and B of the enzyme, have suggested that stromal cells arise from the malignant hematopoietic clone.

In addition to the nontransplantability of marrow stromal cells, our data are against the notion of a common hematopoietic-stromal progenitor. Marrow transplantation with SBA separated grafts provides a unique model to answer whether stromal and hematopoietic cells share a common progenitor. Following BMT, a microenvironment of donor origin could develop through two possible mechanisms: (1) engraftment with a common stromal-hematopoietic progenitor giving rise to both marrow compartments, or (2) engraftment with a transplantable stromal progenitor that gives rise only to stromal cells and not to hematopoietic cells. Conventional marrow grafts containing stromal and hematopoietic progenitors cannot discriminate between these two mechanisms, whereas marrow grafts depleted of stromal progenitors by eliminating the second possibility can be used to test the first mechanism. Our results with stromal depleted marrow grafts showing engraftment with donor hematopoietic cells without the emergence of donor stromal cells, rule out the hypothesis that stromal and hematopoietic cells share a common progenitor.

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Registration Period: January 1, 1988-April 1, 1988
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ONCOLOGY PRACTICE 1988: A PERSPECTIVE

January 31-February 6, 1988
Snowmass, Colorado

For further information, contact: Stephen E. Jones, MD, Charles A. Sammons Cancer Center, Room 4800, Baylor University Medical Center, 3500 Gaston Ave, Dallas, TX 75246. Phone: (214) 820-2021.

CLINICAL HEMATOLOGY AND ONCOLOGY

February 15-17, 1988
San Diego Princess Resort, San Diego, CA

This conference, to be held in San Diego, CA, is designed for hematologists, oncologists, internists, pediatricians, and pathologists interested in the diagnosis and management of hematologic and oncologic disorders. An outstanding faculty from across the country has been assembled to discuss various aspects of these fields, to aid clinicians in sorting out the important from the trivial, and to help identify those recent advances which will withstand the test of time. 24 hours Cat. I.

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The second page should contain an abstract of 200 words or less, summarizing the reason for the study, the methods used, the results, and the major conclusions. Do not include a summary at the end of the paper.

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Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

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1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

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4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

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Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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Color-enhanced scanning electron micrograph of *Pneumocystis carinii.*

Drug of Choice in *PCP*

Bactrim I.V. is the drug of choice for treatment of *Pneumocystis carinii* pneumonia (*PCP*).[1] Please note, though, that AIDS patients may not tolerate or respond to treatment in the same way as non-AIDS patients.

The incidence of side effects—particularly rash, fever and leukopenia—is greatly increased in AIDS patients. Discontinue therapy at the first sign of skin rash or any adverse reaction.

High survival rate after first episodes of *PCP* in AIDS patients

In a multicenter evaluation of 185 AIDS patients who received trimethoprim plus sulfamethoxazole for their first episodes of *PCP*, 58% completed therapy with this combination alone. Among these patients, the rate of immediate survival was 82%.[2]

Immediate survival with TMP/SMX alone
82%
88/107
0%
100%

Adapted from report of a National Heart, Lung and Blood Institute Workshop.[2]

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

ROCHE®

References: 1. *Medical Letter 28*(706):9-16, Jan 31, 1986. **2.** Murray JF, *et al*: *N Engl J Med 310*(25):1682-1688, Jun 21, 1984.

Drug of choice in *PCP*[1]

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

Before prescribing, please consult complete product information, a summary of which follows:

CONTRAINDICATIONS: Hypersensitivity to trimethoprim or sulfonamides; documented megaloblastic anemia due to folate deficiency; pregnancy at term and during the nursing period; infants less than two months of age.

WARNINGS: FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS.

BACTRIM SHOULD BE DISCONTINUED AT THE FIRST APPEARANCE OF SKIN RASH OR ANY SIGN OF ADVERSE REACTION. Clinical signs, such as rash, sore throat, fever, arthralgia, cough, shortness of breath, pallor, purpura or jaundice, may be early indications of serious reactions. In rare instances a skin rash may be followed by more severe reactions, such as Stevens-Johnson syndrome, toxic epidermal necrolysis, hepatic necrosis or serious blood disorder. Perform complete blood counts frequently.

BACTRIM SHOULD NOT BE USED IN THE TREATMENT OF STREPTOCOCCAL PHARYNGITIS. Clinical studies have documented that patients with group A β-hemolytic streptococcal tonsillopharyngitis have a greater incidence of bacteriologic failure when treated with Bactrim than with penicillin.

Bactrim I.V. Infusion contains sodium metabisulfite, which may cause allergic-type reactions in susceptible persons, especially asthmatics or atopic nonasthmatic persons.

PRECAUTIONS:

General: Give with caution to patients with impaired renal or hepatic function, possible folate deficiency (*e.g.*, elderly, chronic alcoholics, patients on anticonvulsants, with malabsorption syndrome, or in malnutrition states) and severe allergies or bronchial asthma. In glucose-6-phosphate dehydrogenase deficient individuals, hemolysis may occur, frequently dose-related.

Local irritation and inflammation due to extravascular infiltration of the infusion have been observed. If these occur, discontinue infusion and restart at another site.

Use in the Elderly: May be increased risk of severe adverse reactions in elderly, particularly with complicating conditions, *e.g.*, impaired kidney and/or liver function, concomitant use of other drugs. Severe skin reactions, generalized bone marrow suppression (see WARNINGS and ADVERSE REACTIONS) or a specific decrease in platelets (with or without purpura) are most frequently reported severe adverse reactions in elderly. In those concurrently receiving certain diuretics, primarily thiazides, increased incidence of thrombocytopenia with purpura reported. Make appropriate dosage adjustments for patients with impaired kidney function (see DOSAGE AND ADMINISTRATION).

Use in the Treatment of Pneumocystis Carinii Pneumonitis in Patients with Acquired Immunodeficiency Syndrome (AIDS): Because of unique immune dysfunction, AIDS patients may not tolerate or respond to Bactrim in same manner as non-AIDS patients. Incidence of side effects, particularly rash, fever, leukopenia, with Bactrim in AIDS patients treated for *Pneumocystis carinii* pneumonitis reported to be greatly increased compared with incidence normally associated with Bactrim in non-AIDS patients.

Laboratory Tests: Perform appropriate culture and susceptibility studies before and throughout treatment. Complete blood counts should be done frequently; if a significant reduction in the count of any formed blood element is noted, discontinue Bactrim. Perform urinalyses with careful microscopic examination and renal function tests during therapy, particularly for patients with impaired renal function.

Drug Interactions: In elderly patients concurrently receiving certain diuretics, primarily thiazides, an increased incidence of thrombocytopenia with purpura has been reported. Bactrim may prolong the prothrombin time in patients who are receiving the anticoagulant warfarin. Keep this in mind when Bactrim is given to patients already on anticoagulant therapy and reassess coagulation time. Bactrim may inhibit the hepatic metabolism of phenytoin. Given at a common clinical dosage, it increased the phenytoin half-life by 39% and decreased the phenytoin metabolic clearance rate by 27%. When giving these drugs concurrently, be alert for possible excessive phenytoin effect. Sulfonamides can displace methotrexate from plasma protein binding sites, thus increasing free methotrexate concentrations.

Drug/Laboratory Test Interactions: Bactrim, specifically the trimethoprim component, can interfere with a serum methotrexate assay as determined by the competitive binding protein technique (CBPA) when a bacterial dihydrofolate reductase is used as the binding protein. No interference occurs if methotrexate is measured by a radioimmunoassay (RIA). The presence of trimethoprim and sulfamethoxazole may also interfere with the Jaffé alkaline picrate reaction assay for creatinine, resulting in overestimations of about 10% in the range of normal values.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Carcinogenesis: Long-term studies in animals to evaluate carcinogenic potential not conducted with Bactrim I.V. Infusion. *Mutagenesis:* Bacterial mutagenic studies not performed with sulfamethoxazole and trimethoprim in combination. Trimethoprim demonstrated to be nonmutagenic in the Ames

BACTRIM™ I.V. Infusion (trimethoprim and sulfamethoxazole/Roche)

assay. No chromosomal damage observed in human leukocytes cultured *in vitro* with sulfamethoxazole and trimethoprim alone or in combination; concentrations used exceeded blood levels of these compounds following therapy with Bactrim. Observations of leukocytes obtained from patients treated with Bactrim revealed no chromosomal abnormalities.

Impairment of Fertility: Bactrim I.V. Infusion not studied in animals for evidence of impairment of fertility. Studies in rats at oral dosages as high as 70 mg/kg trimethoprim plus 350 mg/kg sulfamethoxazole daily showed no adverse effects on fertility or general reproductive performance.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Trimethoprim and sulfamethoxazole may interfere with folic acid metabolism; use during pregnancy only if potential benefit justifies potential risk to fetus. Nonteratogenic Effects: See CONTRAINDICATIONS section.

Nursing Mothers: See CONTRAINDICATIONS section.

Pediatric Use: Not recommended for infants under two months (see CONTRAINDICATIONS section).

ADVERSE REACTIONS: Most common are gastrointestinal disturbances (nausea, vomiting, anorexia) and allergic skin reactions (such as rash and urticaria). **FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS (SEE WARNINGS SECTION).** Local reaction, pain and slight irritation on I.V. administration infrequent. Thrombophlebitis rarely observed.

Hematologic: Agranulocytosis, aplastic anemia, thrombocytopenia, leukopenia, neutropenia, hemolytic anemia, megaloblastic anemia, hypoprothrombinemia, methemoglobinemia, eosinophilia. *Allergic Reactions:* Stevens-Johnson syndrome, toxic epidermal necrolysis, anaphylaxis, allergic myocarditis, erythema multiforme, exfoliative dermatitis, angioedema, drug fever, chills, Henoch-Schoenlein purpura, serum sickness-like syndrome, generalized allergic reactions, generalized skin eruptions, conjunctival and scleral injection, photosensitivity, pruritus, urticaria and rash. Periarteritis nodosa and systemic lupus erythematosus have been reported. *Gastrointestinal:* Hepatitis (including cholestatic jaundice and hepatic necrosis), elevation of serum transaminase and bilirubin, pseudomembranous enterocolitis, pancreatitis, stomatitis, glossitis, nausea, emesis, abdominal pain, diarrhea, anorexia. *Genitourinary:* Renal failure, interstitial nephritis, BUN and serum creatinine elevation, toxic nephrosis with oliguria and anuria, crystalluria. *Neurologic:* Aseptic meningitis, convulsions, peripheral neuritis, ataxia, vertigo, tinnitus, headache. *Psychiatric:* Hallucinations, depression, apathy, nervousness. *Endocrine:* Sulfonamides bear certain chemical similarities to some goitrogens, diuretics (acetazolamide and the thiazides) and oral hypoglycemic agents; cross-sensitivity may exist. Diuresis and hypoglycemia have occurred rarely in patients receiving sulfonamides. *Musculoskeletal:* Arthralgia, myalgia. *Respiratory:* Pulmonary infiltrates. *Miscellaneous:* Weakness, fatigue, insomnia.

DOSAGE AND ADMINISTRATION: CONTRAINDICATED IN INFANTS LESS THAN TWO MONTHS OF AGE. CAUTION—BACTRIM I.V. INFUSION MUST BE DILUTED IN 5% DEXTROSE IN WATER SOLUTION PRIOR TO ADMINISTRATION. DO NOT MIX WITH OTHER DRUGS OR SOLUTIONS. RAPID INFUSION OR BOLUS INJECTION MUST BE AVOIDED.

DOSAGE *(Children and Adults):*

PNEUMOCYSTIS CARINII PNEUMONITIS: Total daily dose is 15 to 20 mg/kg (based on the trimethoprim component) given in three or four equally divided doses every 6 to 8 hours for up to 14 days. One investigator noted that a total daily dose of 10 to 15 mg/kg was sufficient in 10 adults with normal renal function.

SEVERE URINARY TRACT INFECTIONS AND SHIGELLOSIS: Total daily dose is 8 to 10 mg/kg (based on the trimethoprim component) given in two to four equally divided doses every 6, 8 or 12 hours for up to 14 days for severe urinary tract infections and 5 days for shigellosis. Maximum recommended daily dose is 60 ml. *Renal Impaired:* Creatinine clearance above 30 ml/min, give usual dosage; 15-30 ml/min, give one-half the usual regimen; below 15 ml/min, use not recommended.

Method of Preparation: Bactrim I.V. Infusion must be diluted. EACH 5 ML SHOULD BE ADDED TO 125 ML OF 5% DEXTROSE IN WATER. After diluting do not refrigerate; use within 6 hours. If a dilution of 5 ml/100 ml of 5% dextrose in water is desired, use within 4 hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

Multidose Vials: After initial entry into the vial, use remaining contents within 48 hours.

The following infusion systems are satisfactory: unit-dose glass containers; unit-dose polyvinyl chloride and polyolefin containers.

Dilution: ADD EACH 5 ML OF BACTRIM I.V. INFUSION TO 125 ML OF 5% DEXTROSE IN WATER.

NOTE: *When fluid restriction is desirable*, add each 5 ml to 75 ml of 5% dextrose in water; mix just prior to use and administer within two hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

DO NOT MIX BACTRIM I.V. INFUSION—5% DEXTROSE IN WATER WITH DRUGS OR SOLUTIONS IN THE SAME CONTAINER.

ADMINISTRATION: Administer by I.V. infusion over 60 to 90 minutes. Avoid rapid infusion or bolus injection. Do not give intramuscularly.

HOW SUPPLIED: 5-ml ampuls, 5-ml vials and 10-ml vials—boxes of 10; 30-ml and 50-ml multidose vials—boxes of 1. Each 5 ml contains 80 mg trimethoprim (16 mg/ml) and 400 mg sulfamethoxazole (80 mg/ml) for infusion with 5% dextrose in water.

STORE AT ROOM TEMPERATURE (15°-30°C or 59°-86°F). DO NOT REFRIGERATE.

PI 0587

Roche Laboratories
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340 Kingsland Street, Nutley, New Jersey 07110-1199

INTRONA: L'INTERFERON alfa-2b

FORME ET PRESENTATION :
Lyophilisat et solution pour usage parentéral : Boîte unitaire

COMPOSITION :
Lyophilisat : 23,8 mg p. flacon
Interféron alfa-2b 3 millions U.I.
ou 5 millions U.I. ou 10 millions U.I. ou 30 millions U.I.
Excipient : Sodium (hydrogénophosphate de) anhydre,
Phosphate monosodique monohydraté, Acide amino-acétique, Albumine humaine.
Solution : Eau pour usage parentéral : (1 ml).

SORT DU MEDICAMENT :
En sous-cutanée et intramusculaire, les pics sériques sont atteints en 8 et 6 h ; les demi-vies d'absorption en 4 et 3 h et les demi-vies d'élimination en 7 et 6 h.
En intraveineuse, le pic sérique est atteint à la 30e minute (fin de perfusion), la demi-vie d'élimination est d'environ 1 h.
Quelle que soit la voie d'administration, il n'est pas retrouvé d'interféron alfa-2b dans les urines.

PROPRIETES :
Introna, dont le principe actif est l'interféron alfa-2b (interféron alpha, sous type 2) produit par génie génétique, présente ces propriétés qui ont été mises en évidence lors des études précliniques :
1. L'ACTIVITE ANTIPROLIFERATIVE : dose-dépendante.
2. L'ACTIVITE IMMUNOMODULATRICE.
3. L'ACTIVITE ANTIVIRALE.

INDICATIONS :
- Leucémie à tricholeucocytes.
- Mélanome disséminé.
- Sarcome de Kaposi associé au syndrome d'immuno-déficit acquis (SIDA).

CONTRE-INDICATIONS :
- Sensibilité à l'interféron alfa-2b ou à l'un des excipients (albumine humaine entre autres).
- GROSSESSE : en raison de la spécificité d'espèce, les études de tératogénèse et de toxicité fœtale chez l'animal n'ont pas de valeur prévisionnelle pour l'espèce humaine ; il en résulte que l'innocuité de l'interféron alfa-2b pendant la grossesse n'a pu être établie.

MISE EN GARDE :
- Une réaction allergique sévère doit faire interrompre le traitement immédiatement.
- Une surveillance électrocardiographique doit être assurée chez les patients ayant des antécédents cardiaques (infarctus du myocarde et/ou troubles du rythme) et ceux ayant reçu des substances cardiotoxiques.
- Une hypotension peut survenir durant le traitement et jusqu'à 48 h après son arrêt.
- Tout effet secondaire sévère doit conduire à modifier voire arrêter le traitement.

PRECAUTIONS :
- Un état d'hydratation satisfaisant doit être maintenu pendant la durée du traitement.
- Les analgésiques centraux, les hypnotiques et les sédatifs administrés simultanément doivent l'être avec prudence.
- L'innocuité de l'interféron alfa-2b n'a pas été établie ni chez l'enfant ni chez l'adolescent de moins de 18 ans.
- La surveillance biologique porte sur : NFS plaquettes, fonctions hépatique et rénale.

INTERACTIONS MEDICAMENTEUSES :
Incompatibilités majeures :
Aucune substance médicamenteuse ne doit être associée à l'Introna dans la seringue ou le flacon de perfusion.
Ne pas mélanger à des solutions glucosées.

EFFETS INDESIRABLES :
D'une façon générale, les effets indésirables ont un caractère réversible en 72 h et sont dose-dépendants :

Cliniques :
Le plus fréquent est le syndrome pseudo-grippal qui peut être prévenu ou traité par l'administration de paracétamol.
Les autres principaux effets indésirables observés sont :
- Les troubles du système nerveux central.
- Les modifications cardiovasculaires.
- Les troubles gastro-intestinaux.

Biologiques :
leuconeutropénie, thrombopénie, élévation des enzymes hépatiques, troubles de l'hémostase.

MODE D'EMPLOI ET POSOLOGIE :
- La reconstitution doit être effectuée immédiatement avant l'
ploi avec les précautions d'asepsie d'usage.
Prélever la dose appropriée pour injection sous-cutanée
adjonction à 50 ml de soluté isotonique de NaCl pour adm
tration intraveineuse (perfusion de 30 minutes).
- Aucune substance ne doit être associée à Introna dan
seringue ou le flacon de perfusion.

Les posologies recommandées sont les suivantes :
- *Leucémies à tricholeucocytes*
2 millions d'Unités Internationales/m²/jour par voie sous-cuta
3 fois par semaine, 1 jour sur 2.
- *Mélanome disséminé*
5 à 10 millions d'Unités Internationales/m²/jour par voie s
cutanée, 3 fois par semaine, 1 jour sur 2.
- *Sarcome de Kaposi associé au SIDA*
50 millions d'Unités Internationales/m²/jour en perfusion
30 minutes, 5 jours consécutifs, 1 semaine sur 2 (intervalle
9 jours entre 2 cures).

Conservation : A conserver au réfrigérateur (de 2 à 8 °C).
Après reconstitution, conserver au maximum 24 h entre 2
30 °C.

Une demande a été faite pour étendre la validité de conserva
au réfrigérateur à 36 mois et à température ambiante à 18 m
conformément aux résultats des études de stabilité conduites
plusieurs dizaines de lots et approuvés par l'expert analyste.

SURDOSAGE :
Aucun cas de surdosage n'a été rapporté à ce jour, bien que doses de 200 millions d'Unités Internationales aient administrées.

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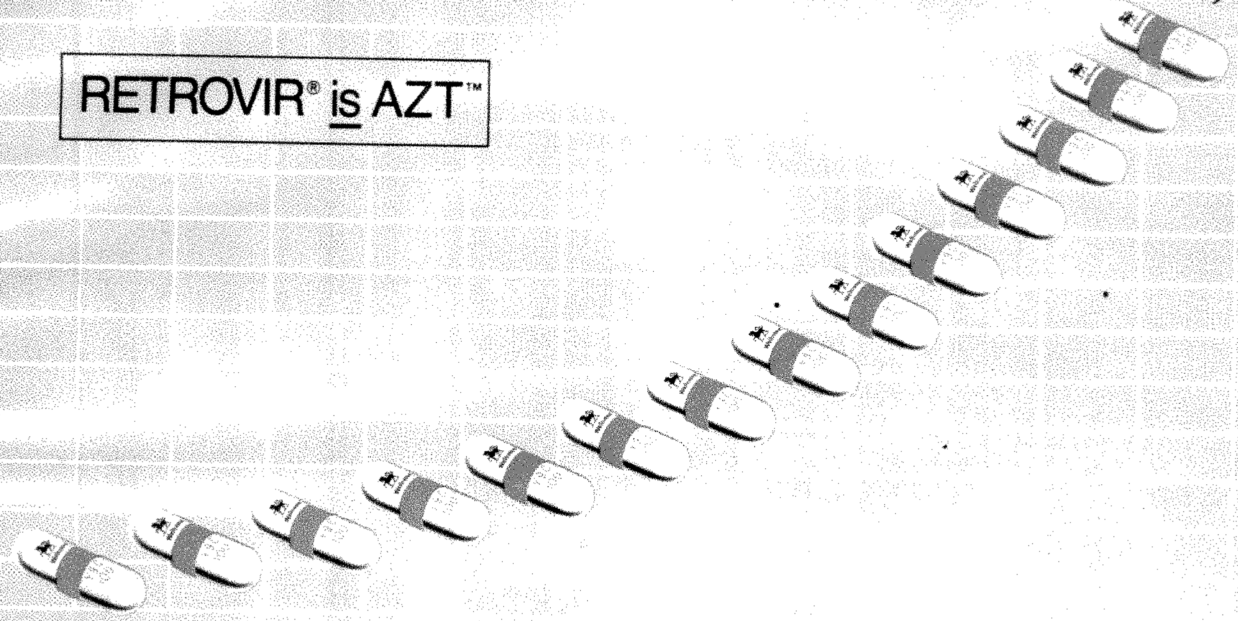

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IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OI'S) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than 200/mm^3 in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 160 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss (> 15 lbs or > 10% of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 cells/mm^3 at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of < 750/mm^3. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count < 1000/mm^3 or hemoglobin < 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 µg/ml and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/TK$^{+/-}$ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 µg/ml). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 µg/ml and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 µg/ml and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 µg/ml.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine; 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly related to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table.

	Pretreatment CD4 (T4) Levels			
	< 200/mm^3		> 200/mm^3	
Abnormality	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia (< 750/mm^3)	47%	10%	10%	3%
Anemia (Hgb < 7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

		Pretreatment CD4 (T4) Levels			
		< 200/mm^3		> 200/mm^3	
Abnormality	% Decrease from Baseline	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	> 50%	55%	19%	40%	13%
Anemia	> 25%	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4	NERVOUS		
Fever	16	12	Dizziness	6	4
Headache	42	37	Insomnia	5	1
Malaise	8	7	Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4	SKIN		
GI Pain	20	19	Rash	17	15
Nausea	46	18	SPECIAL SENSES		
Vomiting	6	3	Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.
Cardiovascular: vasodilation.
Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.
Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.
Nervous: anxiety, confusion, depression, emotional lability, nervousness, syncope, loss of mental acuity, vertigo.
Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.
Skin: acne, pruritus, urticaria.
Special senses: amblyopia, hearing loss, photophobia.
Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of < 7.5 g/dL or reduction of > 25% of baseline) and/or significant granulocytopenia (granulocyte count of < 750/mm^3 or reduction of > 50% from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.

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Transplantation Immunologist. A scientist (Ph.D. or MD) is being sought to join the BMT program to collaborate in developing investigative approaches to current controversies in bone marrow transplantation. The candidate should have significant research experience and demonstrated scholarly productivity.

Oncologist. A pediatric hematologist/oncologist (BC/BE) with interests and expertise in both clinical and laboratory investigation, preferably in the biology of solid tumors.

Selected candidates would receive appropriate academic appointments in the Department of Child Health and Development, George Washington University School of Medicine and Health Sciences, and salaries commensurate with qualifications and experience. Candidates should send letter of interest and CV to: Gregory H. Reaman, M.D.
Chairman, Hematology/Oncology
Children's Hospital National Medical Center
111 Michigan Avenue, N.W.
Washington, D.C. 20010

LEUKEMIA RESEARCH FOUNDATION GRANTS

The Leukemia Research Foundation, Inc. announces funds are available to support research in the field of leukemia. Currently two types of grants are being funded; research grants, and postdoctoral fellowships.

The following guidelines apply:

1. Maximum limit is $35,000 for research grants, and $20,000 for postdoctoral fellowships.
2. Grants and fellowships are for a one-year period.
3. Institution of affiliation must provide both a report of the results of the research and a financial report.
4. No funds shall be applied to institutional overhead (indirect costs).
5. Preference will be given to researchers new to this field.
6. Deadline for receipt of completed grant applications is February 22, 1988.

For further information and for applications, contact:
Hollis R. Brownstein, Chairman
Medical Advisory Committee
Leukemia Research Foundation, Inc.
899 Skokie Blvd., Suite LL14
Northbrook, Illinois 60062
Phone: 1-312/480-1177

HUMAN GENETICS

Bruce L. Evatt, M.D., Director, the Division of Host Factors, Center for Infectious Diseases, Centers for Disease Control, invites applications for a research scientist in molecular genetics. Individuals with research interests in hematopoiesis or stem cell differentiation are particularly encouraged to apply. Responsibilities include the development and execution of program plans in coordination with the Division Director. A fully equipped laboratory and technical staff are provided. Prefer candidates with a Ph.D. and/or M.D. and a minimum of two years postdoctoral experience in molecular genetics. Salary ranges from $38,000-$72,000 depending on qualifications and personnel systems. Applications including curriculum vitae, a statement of future research interests, and the names with addresses of three references should be sent to: Sharlyn L. Wilkinson, Personnel Management Specialist, CDC, Bldg. 1, Room 1058, Atlanta, GA 30333. Applications must be received by March 15, 1988 to receive consideration.

CDC is an equal opportunity employer and has a smoke-free work environment.

Georgia—The Section of Hematology/Oncology, Department of Pediatrics, Medical College of Georgia is recruiting a fourth faculty member at the assistant/associate professor level. Board certification in pediatrics and subboard eligibility/certification in pediatric hematology/oncology required. Responsibilities include patient care, teaching, and research. The ideal candidate would have research interest in leukocyte or coagulation disorders. Contact P. Charlton Davis, M.D., Chief, Pediatric Hematology/Oncology, Medical College of Georgia, Augusta, GA 30912-3770, Phone (404) 721-3626. EOE/AAP

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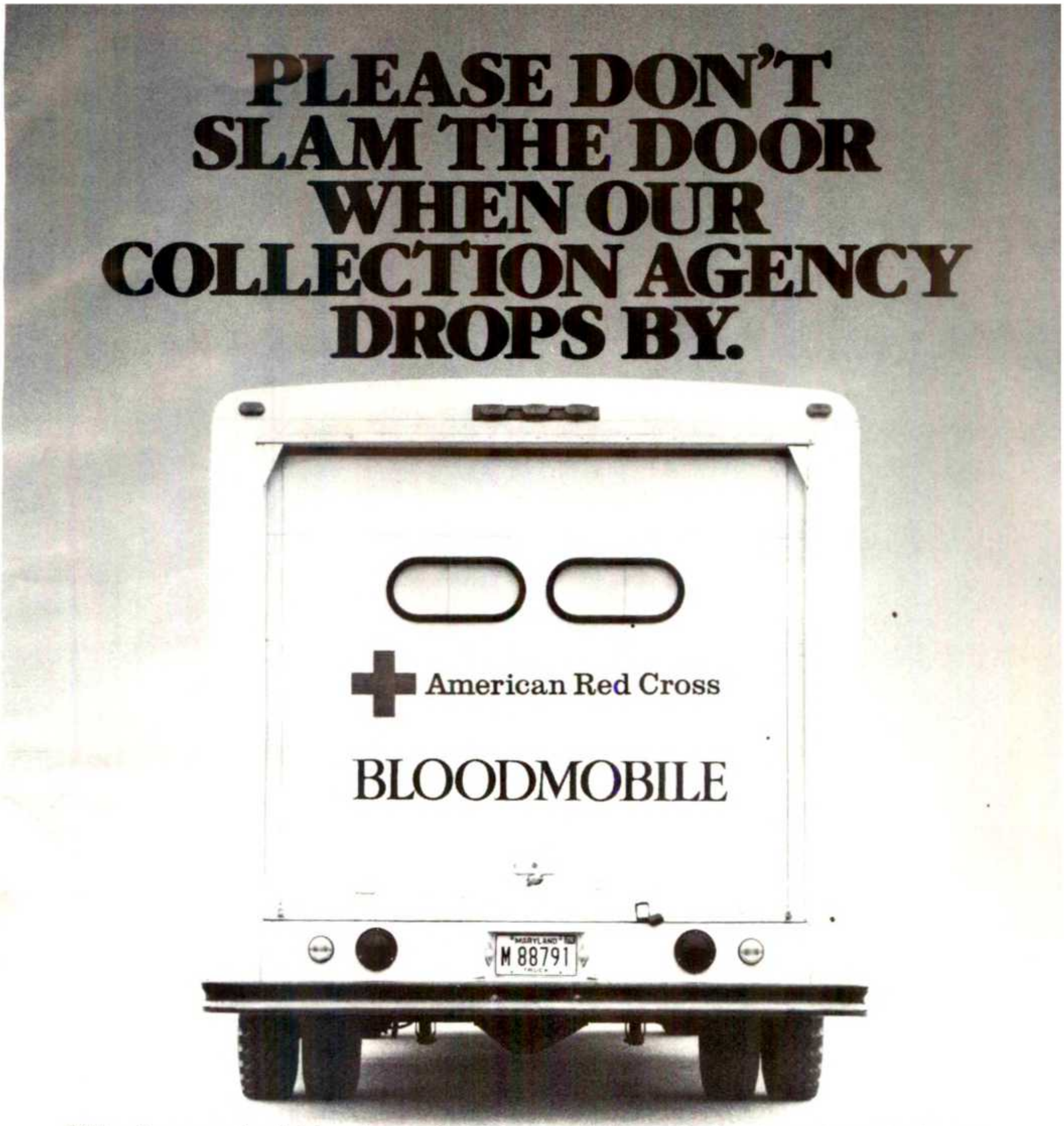

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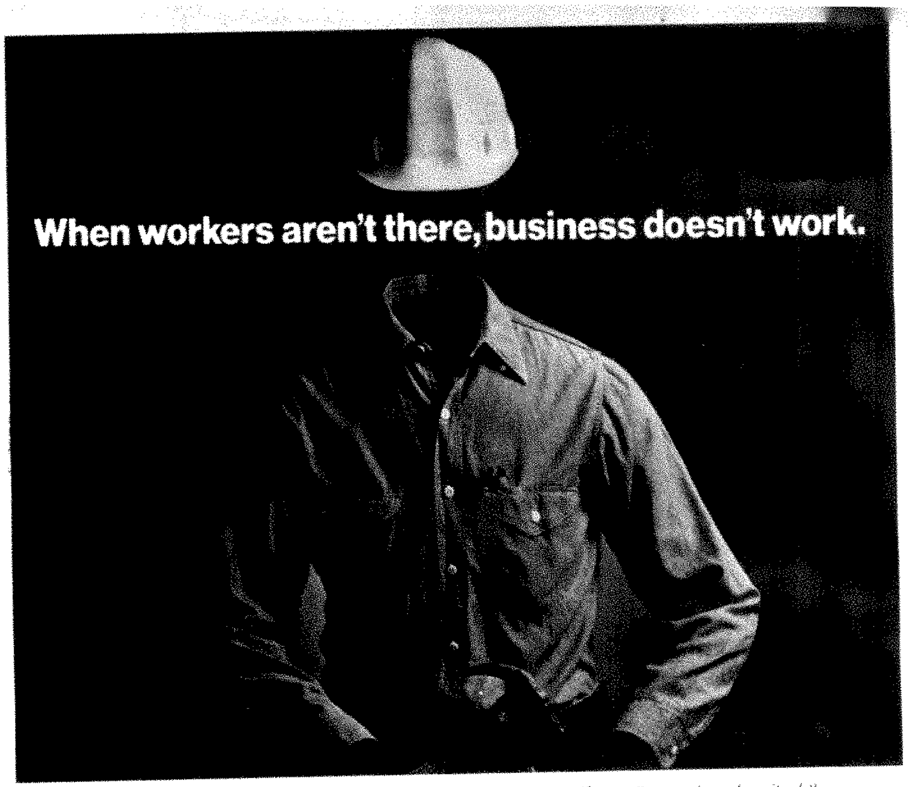
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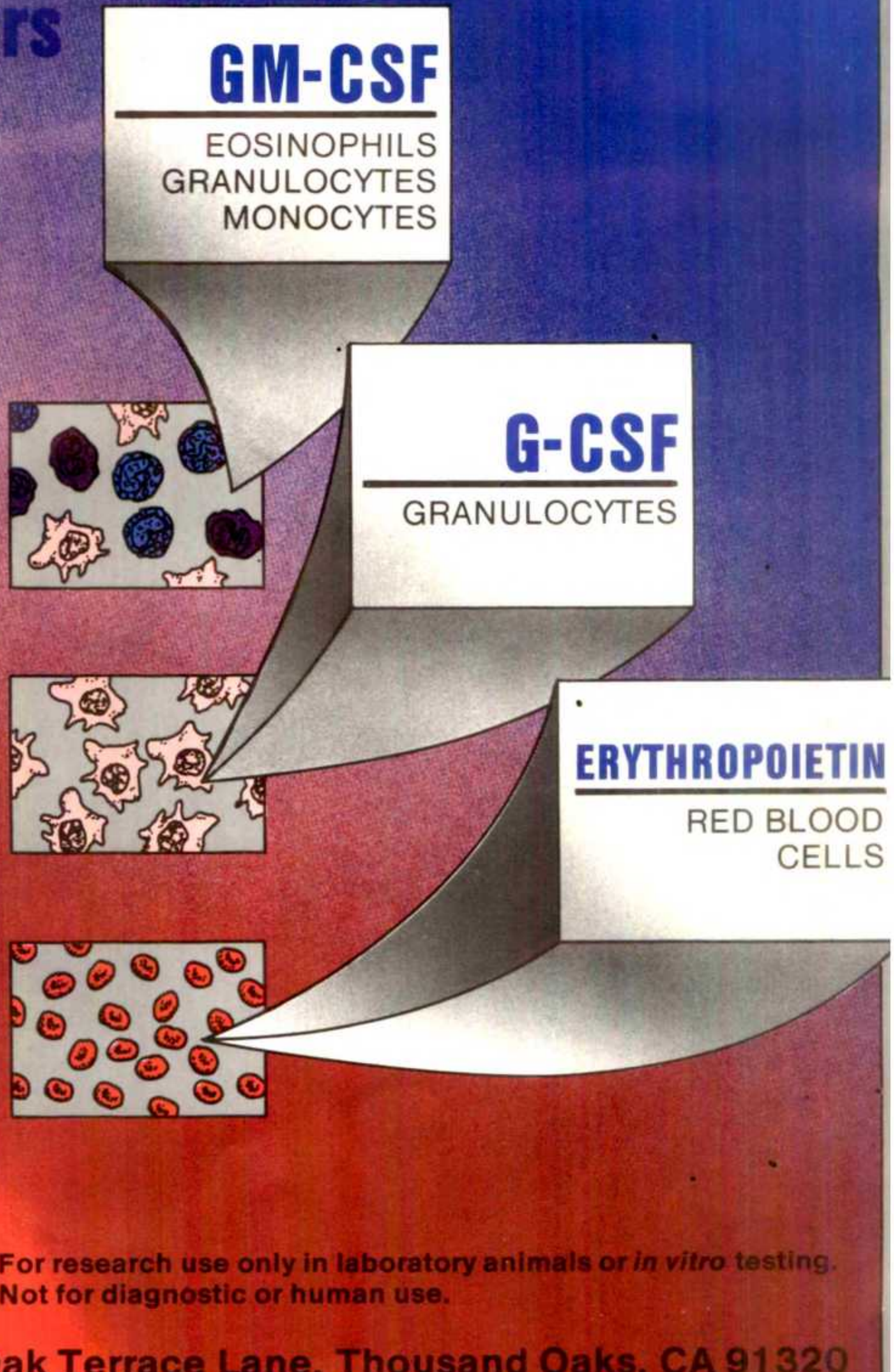

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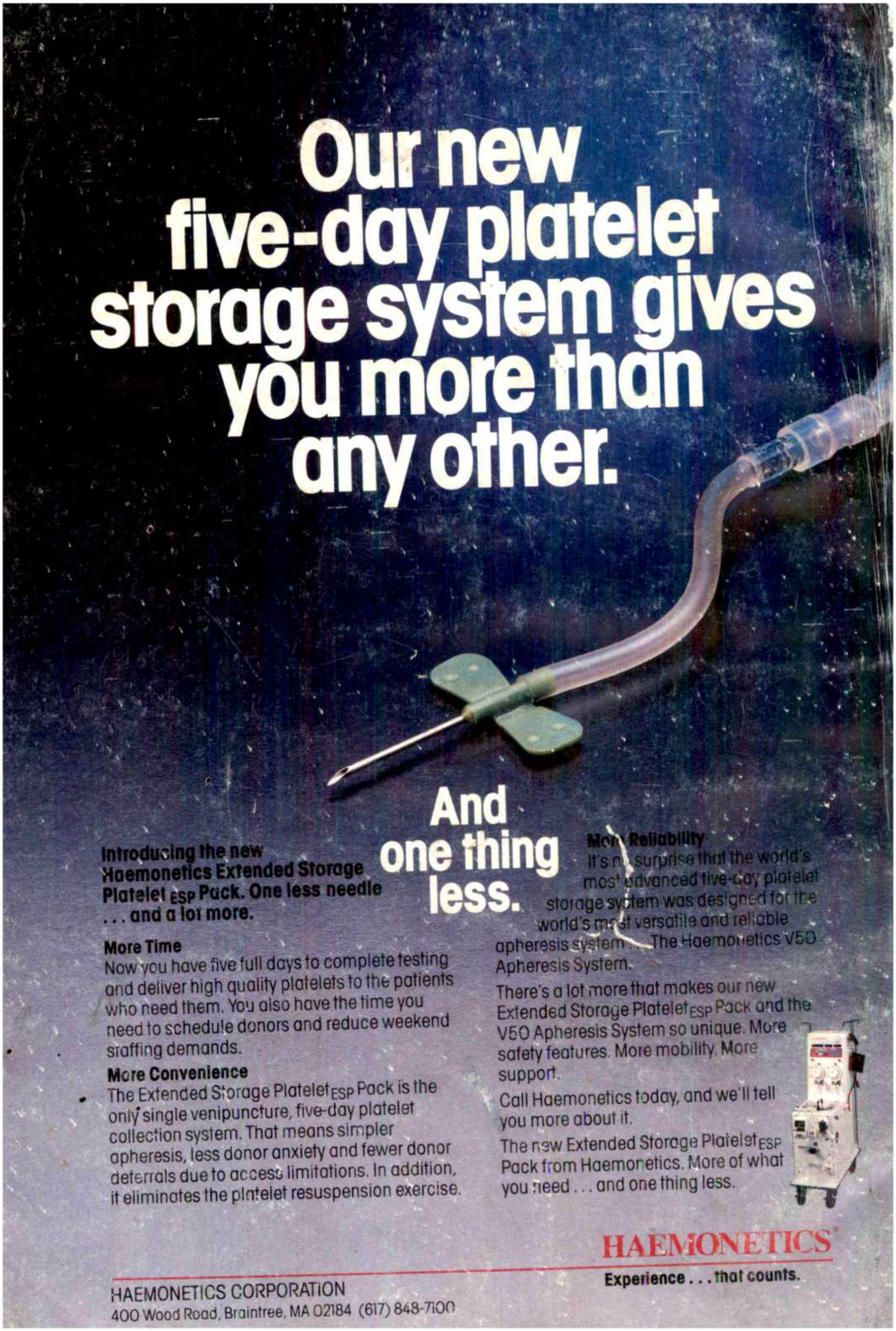

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